

ON THE INTESTINAL TURNOVER OF PANCREATIC AND GRANULOCYTIC SERINE PROTEASES AND PROTEASE INHIBITORS

with special reference to severe ulcerative colitis

Måns Bohe



Malmö 1986



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ON THE INTESTINAL TURNOVER OF PANCREATIC AND GRANULOCYTIC
SERINE PROTEASES AND PROTEASE INHIBITORS WITH SPECIAL
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Abstract The intestinal turnover of protease inhibitors and pancreatic and granulocytic serine proteases was studied in healthy subjects and patients with ulcerative colitis (u.c.) by immunochemical analyses of ileostomy contents, faeces and plasma. Gastrointestinal mucosa specimens were analysed with the unlabelled enzyme method for the presence of immunoreactive (i.r.) pancreatic proteases and i.r. pancreatic secretory trypsin inhibitor (PSTI). Turnover studies with ^{131}I - and ^{125}I -trypsin were performed in man and dog. Ileostomy content was found to contain 50-200 mg/24 hours of the different extractable i.r. pancreatic endoproteases. Faecal extracts from healthy subjects contained no proteolytic activity and only trace amounts of i.r. pancreatic and granulocytic endoproteases. Faecal extracts from patients with acute u.c. contained a marked proteolytic activity and high levels of i.r. anionic trypsin, i.r. cationic elastase and i.r. granulocytic elastase. Faecal extracts from patients with acute u.c. contained, in contrast to those from healthy subjects, high levels of $\alpha_2\text{-M}$, $\alpha_1\text{-PI}$ and ACHY. These protease inhibitors were, for the major part modified and unable to bind added proteases, and for the minor part in complex formation with the granulocytic proteases elastase and neutral protease. The plasma level of $\alpha_2\text{-M}$ was decreased in patients with acute u.c. due to consumption of released granulocytic proteases. Paneth cells in normal and metaplastic mucosa contained i.r. anionic and cationic trypsin and i.r. PSTI, but no chymotrypsin or elastase-like i.r. material. The goblet cells in the small intestine contained PSTI-like i.r. material but no endoprotease-like i.r. material. No pancreatic resecretion of ^{131}I intravenously injected ^{125}I -trypsin was found. After intraduodenal instillation of ^{131}I -trypsin in man all recovered radioactivity in plasma were in the form of free ^{131}I , thus a deiodination mechanism exists in the intestine.		
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ABBREVIATIONS

α_1 -PI	=	alpha ₁ -proteinase inhibitor
α_2 -M	=	alpha ₂ -macroglobulin
ACHY	=	alpha ₁ -antichymotrypsin
DFP	=	di-iso-propyl-fluorophosphate
i.r.	=	immunoreactive
PSTI	=	pancreatic secretory trypsin inhibitor

This thesis is based on the following papers,
which will be referred to by their Roman numerals

- I DETERMINATION OF IMMUNOREACTIVE TRYPSIN, PANCREATIC ELASTASE AND CHYMOTRYPSIN IN EXTRACTS OF HUMAN FECES AND ILEOSTOMY DRAINAGE.
Bohe M, Borgström A, Genell S, Ohlsson K.
Digestion 1983, 27:8-15
- II METABOLISM OF ^{131}I -LABELLED HUMAN PANCREATIC CATIONIC TRYPSIN AFTER INTRADUODENAL ADMINISTRATION.
Bohe M, Borgström A, Genell S, Ohlsson K.
Digestion. In press.
- III FATE OF INTRAVENOUSLY INJECTED TRYPSIN IN DOG WITH SPECIAL REFERENCE TO THE EXISTENCE OF AN ENTERO-PANCREATIC CIRCULATION.
Bohe M, Borgström A, Genell S, Ohlsson K.
Digestion 1984, 29:158-163.
- IV TRYPSIN-LIKE IMMUNOREACTIVITY IN HUMAN PANETH CELLS.
Bohe M, Borgström A, Lindström C, Ohlsson K.
Digestion 1984, 30:271-275.
- V PANCREATIC ENDOPROTEASES AND PANCREATIC SECRETORY TRYPSIN INHIBITOR IMMUNOREACTIVITY IN HUMAN PANETH CELLS.
Bohe M, Borgström A, Lindström C, Ohlsson K.
J Clin Pathol. In press.
- VI IMMUNOREACTIVE PANCREATIC SECRETORY TRYPSIN INHIBITOR IN SERA FROM PATIENTS WITH ACUTE ULCERATIVE COLITIS.
Bohe M.
Submitted for publication
- VII PANCREATIC AND GRANULOCYTIC ENDOPROTEASES IN FAECAL EXTRACTS FROM PATIENTS WITH ACTIVE ULCERATIVE COLITIS.
Bohe M.
Submitted for publication
- VIII PROTEASE INHIBITORS IN PLASMA AND FAECAL EXTRACTS FROM PATIENTS WITH ACTIVE INFLAMMATORY BOWEL DISEASE.
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INTRODUCTION

Ulcerative colitis is a chronic inflammatory disease of the colon and rectum of an unknown aetiology (Kirsner & Shorter 1982a,b). The disease usually begins in the rectum and extends proximally and continuously to involve the whole or part of the remaining colon. In some patients it may involve the entire colon from the onset of the disease. Ulcerative colitis is usually associated with recurrent attacks of bloody diarrhoea, cramping abdominal pain, anorexia and weight loss with complete freedom from symptoms in the interim (Truelove & Witts 1955, Edwards & Truelove 1963, 1964). Severe attacks of ulcerative colitis may lead to acute dilatation of the colon and/or perforation of the colonic wall (Edwards & Truelove 1964, Goligher et al 1970). The diagnosis is based on clinical signs, sigmoidoscopic appearance, double-contrast barium enema and colonoscopy in combination with histopathological examination of mucosal specimens. Infectious colitis is excluded by negative faecal cultures.

Macroscopically, in active disease there is mucosal hyperemia, granularity, ulceration and bleeding. Histologically quiescent disease is characterized by reduced numbers of mucosal crypts and a frequent occurrence of Paneth cells. Acute ulcerative colitis is characterized by intense mucosal inflammation, aggregates of neutrophils forming crypt abscesses which can form large intramucosal abscesses completely destroying the crypt epithelium and extending to the underlying lamina propria, depletion of goblet cell mucin and ulceration. The changes are found mainly in the mucosa but in fulminating disease deeper layers of the colonic wall are affected. In severe acute ulcerative colitis the serosal side is often free from inflammatory changes although there is an extensive ulceration in the mucosa exposing the underlying submucosa and muscularis. The ulcerations are covered by a mixture of blood, mucus, pus and liquid faeces (Morson & Dawson 1979). Obviously pancreatic as well as granulocytic proteases may be involved in the tissue destruction.

The intense inflammatory process is accompanied by marked alteration of some serum proteins (Bicks 1959, de Dombal 1968, Weeke & Jarnum 1971, Marner et al 1975). Levels of the acute-phase proteins α_1 -proteinase inhibitor (α_1 -PI), α_1 -antichymotrypsin (ACHY) and orosmucoid are increased. The albumin level is decreased due to increased catabolic rate (Bendixen et al 1970). The levels of immunoglobulin are unchanged (Weeke & Jarnum 1971). An unexplained decreased serum level of α_2 -macroglobulin (α_2 -M), one of the major plasma protease inhibitors, has been reported in patients with severe ulcerative colitis (Weeke & Jarnum 1971, Marner et al 1975, Brown et al 1980).

A drastic release of proteases in acute ulcerative colitis is apparent from the widespread tissue destruction and is also indicated by the observed low plasma level of α_2 -M in this disease. The identity of the proteases mainly responsible for the tissue digestion is, however, not known.

PURPOSE OF THE PRESENT INVESTIGATION

The present series of investigations was undertaken in order to study the protease/protease-inhibitor balance in patients with severe ulcerative colitis. It was, however, first necessary to study the normal amounts and metabolism of the dominating pancreatic and granulocytic proteases and the different protease inhibitors in the intestinal tract.

Answers were sought to the following questions.

What are the normal levels of pancreatic and granulocytic endoproteases in ileostomy contents and faeces?

Does an enteropancreatic recirculation of trypsin exist?

Are there any pancreatic endoproteases present in or bound to the intestinal mucosa?

Is there a proteolytic activity in faeces from patients with acute colitis and if so, which proteolytic enzymes are involved?

Are there protease inhibitors in faeces from patients with acute colitis and, if so, are there residual inhibiting capacity and signs of proteases/protease-inhibitor interaction?

BACKGROUND

Pancreatic proteases

One of the main sources of proteolytic enzymes in the gastrointestinal tract is the pancreatic gland. The pancreas, having the highest protein turnover per gram tissue of all human organs (Kukral et al 1965) produces about 1 litre of pancreatic juice per day containing about 5 g proteases (Borgström 1979). The proteolytic enzymes in pancreatic juice are exo- and endopeptidases. The exopeptidases hydrolyse peptide bonds from the polypeptide terminals and endopeptidases hydrolyse peptide bonds within the polypeptides. The pancreatic endopeptidases, as well as plasmin, thrombin and the granulocytic enzymes elastase, cathepsin G and neutral protease, are serine proteases (Walsh & Wilcox 1970). These enzymes are characterized by a serine residue at the active site and are irreversibly inhibited by di-iso-propyl-fluorophosphate (DFP) (Walsh & Wilcox 1970). The endoproteases in human pancreatic juice consist of two trypsinogens (Guy et al 1978), two chymotrypsinogens (Feinstein et al 1974) and two proelastases (Mallory & Travis 1974, Ohlsson & Olsson 1976, Largman et al 1976). These proenzymes are secreted together with the pancreatic secretory trypsin inhibitor (Kazal et al 1948). The activating process takes part in the duodenum where trypsinogen is converted to its active form, trypsin, by enterokinase (Abita et al 1969). The other proenzymes are then converted into active enzymes by limited proteolysis by trypsin (Walsh 1970, Wilcox 1970, Shotton 1970). In serum the pancreatic endoproteases are inhibited mainly by α_2 -M and α_1 -PI (Ohlsson 1974).

The intestinal content of trypsin and chymotrypsin over the entire length of the small intestine was measured with enzymatic methods by Borgström et al (1957). They found a decreasing concentration of these enzymes distally and postulated that the digestive enzymes are partially degraded or inactivated in the small intestine. In a 24-hour collection of ileostomy contents about 150 mg trypsin and 250 mg chymotrypsin were recovered by Roy et al (1967). Two thirds of the proteolytic activity in ileostomy contents were found to be due

to trypsin and chymotrypsin (Goldberg et al 1968a). By comparing homogenates and supernatant fluids after centrifugation of the ileostomy contents about 1/3 of the recovered trypsin activity and half of the chymotrypsin activity were found to be bound to insoluble debris (Roy et al 1967). Bovine trypsin and chymotrypsin incubated with ileal mucosa homogenates were found to be bound to intact mucosa cells (Goldberg et al 1968b, 1969). Fresh large bowel mucosa homogenates inhibit the enzymatic activity of added trypsin and chymotrypsin suggesting the occurrence of an inhibitor of these two enzymes in the large bowel mucosa (Goldberg et al 1969). The bacterial flora is important in the degradation of pancreatic endoproteases (Genell et al 1976, Genell & Gustafsson 1977, Borgström et al 1977). Using both enzymatic and immunochemical methods considerable amounts of the different pancreatic endoproteases are demonstrated in supernatant fluids from faecal homogenates from germfree rats and conventional rats after antibiotic treatment, while only trace amounts are present in supernatant fluids from faecal homogenates from conventional rats (Genell et al 1976, Genell & Gustafsson 1977). Impaired degradation in the colon of trypsin and elastase after antibiotic treatment has also been found in man (Borgström et al 1977). Increased levels of tryptic activity, compared with healthy controls, have been found in faecal homogenates (Merwe & Mol 1982) and supernatant fluids from faecal samples from patients with Crohn's disease (Bergstrand et al 1981). No studies seem to have been performed on patients with ulcerative colitis.

It has been proposed that the pancreatic proteases play a role in the pathogenesis of the mucosal destruction in ischemic enteropathy (Bounous 1982). Mucosal lesions have been found in the small bowel after ischemia (Bounous et al 1965, Bounous 1982). These lesions are minimized by previous ligation of the pancreatic duct (Bounous et al 1965) or by previous inhibition of the different pancreatic endoproteases by aprotinin (Bounous et al. 1966), turkey ovomucoid (Bounous et al 1979) and soybean trypsin inhibitor (Parks et al 1985). On the other hand, an intraluminal injection of trypsin or elastase aggravates these lesions (Bounous et al 1977). It has also been

found that the mucosal ulcerations seen after radiation therapy are minimized after exclusion of the pancreatic proteases by previous pancreatectomy or ligation of the pancreatic duct (Morgenstern & Hiatt 1967, Morgenstern et al 1970).

Recently an enteropancreatic circulation of active pancreatic proteases as a conservation mechanism has been suggested by some authors (Götze & Rothman 1975, Heinrich et al 1979, Lake Bakaar et al 1980) but rejected by others (Levitt et al 1981, Rohr et al 1981). The pancreatic endoproteases are also present in the blood but only in trace amounts. In serum the immunoreactive cationic trypsin consists of trypsinogen with a normal concentration of 25-50 µg/l (Borgström & Ohlsson 1976, Geokas et al 1979).

Granulocytic proteases

The normal daily turnover of granulocytes (about 10^{11} cells) represents about 1 g of proteolytic enzymes. During inflammatory processes these figures are drastically increased (Ohlsson & Olsson 1977a). The granulocytes contain the metalloproteinase collagenase (Lazarus et al 1968) and the serine proteases elastase (Ohlsson & Olsson 1974, Baugh & Travis 1976), neutral protease (Ohlsson & Olsson 1973) and cathepsin G (Feinstein & Janoff 1975). The serine proteases are located in the azurophil granule (Ohlsson et al 1977) and collagenase in the specific granule (Murphy et al 1977) of the granulocytes. During phagocytosis the granulocyte proteases are at least partly released from the cells (Ohlsson & Olsson 1977a) resulting in a local increase in proteolytic enzymes which may lead to tissue destruction (Ohlsson 1976). The granulocyte serine proteases can digest important connective tissue components such as collagen, fibrin, fibronectin and proteoglycans. Granulocytic elastase can, in addition, degrade elastin, an activity accelerated by cathepsin G (Boudier et al 1981). The granulocyte proteases, in addition, probably interact with the cascade systems and also with the functions of several cell types (for a review see Ohlsson 1985). The proteolytic activity on the complement factors C3 and C5 results in chemotactically active substances which further increase the influx

of granulocytes (Wenge & Olsson 1975, Johnson et al 1976). The activity of the proteolytic enzymes is balanced and counteracted by a series of protease inhibitors present in plasma and tissue fluids. After incubation of trace amounts of pure granulocytic proteases and serum, 90% of the granulocyte elastase is bound to α_1 -PI and 10 % to α_2 -M (Ohlsson & Olsson 1977b). The corresponding figures for neutral protease are 50% to α_1 -PI and 50% to α_2 -M (Ohlsson & Olsson 1974) and for cathepsin G 2% to α_1 -PI, 86% to α_2 -M and 12% to ACHY (Ohlsson & Åkesson 1976).

Protease inhibitors

Pancreatic secretory trypsin inhibitor (PSTI)

PSTI was originally isolated from the pancreatic gland (Kazal et al 1948) and later also from pancreatic juice (Greene et al 1966, Eddeland & Ohlsson 1978a). The molecular weight is 7,000 daltons. It constitutes 0.1-0.8% of the total protein content in pancreatic juice. The ratio of PSTI to trypsinogen (0.02-0.03) in pancreatic juice is fairly constant even after stimulation of the pancreatic gland (Eddeland & Wehlin 1978). Increased serum levels are found in patients with acute pancreatitis (Eddeland & Ohlsson 1978b, Otsuki et al 1984). PSTI forms complexes with trypsin and these complexes are dissociated in serum milieus, where trypsin is taken over mainly by α_2 -M. Free PSTI is eliminated from serum with a half-life of 7 minutes (Marks & Ohlsson 1983). The main function of PSTI is thought to be the protection of the pancreatic gland from the effects of premature activation of trypsinogen within the gland (Greene et al 1966, Schweitz et al 1973). With sensitive immunological methods PSTI can be detected in normal serum at a concentration of 10 μ g/l (Eddeland & Ohlsson 1978b).

Plasma protease inhibitors

The plasma protease inhibitors α_1 -PI, α_2 -M and ACHY constitute about 10% of the total plasma protein content and are responsible for more than 90% of the protease inhibitory capacity in plasma (Laurell & Jeppsson 1975).

α_1 -Proteinase inhibitor (α_1 -PI) has a molecular weight of about 54,000 daltons and is synthesized in the liver (Laurell & Jeppsson 1975). The normal plasma concentration is 1.3 g/l. α_1 -PI is an acute-phase protein and during inflammatory diseases and after surgical trauma increased serum levels are found (Aronsen et al 1972). α_1 -PI inhibits both the esterolytic and the proteolytic activity of the serine proteases by forming complexes in a molar ratio of 1:1. Proteases bound to α_1 -PI are thought to be at least partially transferred to α_2 -M and subsequently eliminated from the circulation by the liver (Ohlsson & Laurell 1976, Balldin et al 1978, Beatty et al 1982). After the dissociation of α_1 -PI protease complexes, α_1 -PI does not retain its inhibitory capacity and has an altered electrophoretic mobility (Ohlsson & Tegner 1975, Stockley et al 1982).

α_1 -PI can be detected in faecal extracts and faecal α_1 -PI has been shown to be useful in measuring the intestinal protein loss in inflammatory bowel disease (Karbach et al 1983, Meyers et al 1985) and in screening cases with suspected protein-losing enteropathy (Crossley & Elliot 1977, Bernier et al 1978).

α_2 -Macroglobulin (α_2 -M), an inhibitor of all types of endoproteases, has a molecular weight of about 725,000 daltons and a mean serum concentration of 2.0 g/l. There is usually a wide variation between individuals. α_2 -M is produced mainly in the liver (Laurell & Jeppsson 1975). α_2 -M is also produced by alveolar macrophages (White et al 1980) and fibroblasts (Cassiman et al 1980). The serum level of α_2 -M is stable in most diseases except in sepsis (Gallimore et al 1980), pancreatitis (McMahon et al 1984, Lason & Ohlsson 1984), total severe colitis (Weeke & Jarnum 1971, Brown et al 1980) and after major bone surgery (Dickson & Alper 1974), where low serum levels have been found. α_2 -Macroglobulin inhibits the different pancreatic endoproteases, the granulocytic proteases, plasmin, thrombin and kallikrein, by complex formation. Complex formation with α_2 -M is thought to be an irreversible entrapment of the active protease caused by a

conformational change in the inhibitor molecule (Barrett & Starkey 1973). Later studies have shown the protease to be covalently bound to the inhibitor (Salvesen & Barrett 1980). The active site of the protease is not blocked by α_2 -M and low-molecular-weight proteins can reach the protease. Thus, the proteases bound to α_2 -M lose their proteolytic activity but retain their esterolytic activity on low molecular substrates (Barrett & Starkey 1973). After complex formation, α_2 -M is eliminated from the circulation by the reticulo-endothelial cell system (Ohlsson & Laurell 1976, Balldin et al 1978). The half-life of these complexes in serum is about 10 minutes. Later studies in rats also suggest that the hepatocytes play an important role in the elimination of these complexes (Davidsen et al 1985). The major part of the protease- α_2 -M complexes found in inflammatory processes may, however, never reach the circulation. They are probably eliminated by macrophage-like cells locally and in adjacent lymph nodes (Ekerot & Ohlsson 1982, Ekerot et al 1982).

Antichymotrypsin (ACHY) has a molecular weight of 69,000 daltons. The normal serum concentration is 0.5 g/l. ACHY is an acute-phase protein. Increased serum levels are found during bacterial infections and after surgical trauma (Aronsen et al 1972, Laurell & Jeppsson 1975). Antichymotrypsin inhibits chymotrypsin and cathepsin G by complex formation in a 1:1 molar concentration. The inhibition of cathepsin G may be a physiologically important property (Ohlsson & Åkesson 1976). Antichymotrypsin has been assumed to be involved in the defence mechanism of mucous membranes (Ryley & Brogan 1973).

PRESENT INVESTIGATIONS

Comments on subjects and methods

Subjects

The diagnosis ulcerative colitis was established by the findings of proctoscopy, colonoscopy or double-contrast enema of the large bowel in combination with the histological findings of biopsies from the large bowel. The activity of the disease was classified according to Truelove & Witts (1955).

In all subjects studied a careful history of antibiotic treatment during the last year was obtained, as recent antibiotic treatment has been found to give increased amounts of extractable immunoreactive proteases in faeces (Genell & Gustafsson 1977, Borgström et al 1977).

Methods

Faeces and ileostomy samples. Analyses were done on spot samples from faeces or ileostomy contents. Measurements on spots samples have been found to reflect the total faecal content (Walker et al 1971). Polyethyleneglycol (PEG 4000) was used as a marker for calculation of the total faecal mass (Malawer et al 1967). Immunochemical methods, as used in the present series of investigations, cannot be used on homogenates. Therefore supernatant fluids, from faecal or ileostomy contents extracted in 5 volumes of ice-cold saline and centrifuged for 30 min at 4°C at 39,000 x g, were used. A part of each sample was immediately incubated with di-isopropyl-fluorophosphate (DFP), a serine protease inhibitor, to a final concentration of 10 mmol/l. This was done to prevent autodigestion during storage.

Quantitative analyses. In most studies on pancreatic proteases in faecal samples, enzymatic methods have been used (Borgström et al 1957, Goldberg et al 1968a, 1969, Dürr et al 1978). A drawback using enzymatic methods is the lack of specificity e.g. the substrate used can be cleaved by more than one enzyme, and furthermore, some bacteria also produce

elastase, trypsin and chymotrypsin-like enzymes (Johnson et al 1967, Pacaud & Richaud 1975, Pacaud 1976, Pacaud et al 1976). Therefore, a specific and at the same time sensitive method was sought, and immunochemical methods were found to be useful. In order to obtain reliable results with immunochemical methods, certain requirements must be fulfilled. The immunoreactivity of the antigen in the standards must be identical to the measured immunoreactivity of the antigen in the samples. This can be ensured by comparing the recovery after dilution of standard and samples. Parallel dilution curves for standard and samples indicate similar immunoreactivities. Biological samples may also contain degradation products. Chromatographic separation gives information on the molecular size of the measured immunoreactive enzyme and this is of importance for the reliability of the quantitative data.

Radioimmunoassays were used for the quantification of the different pancreatic endoproteases, granulocytic elastase and PSTI (Borgström & Ohlsson 1976, Largman et al 1978, Borgström et al 1980, Geokas et al 1979, Ohlsson & Olsson 1978, Eddeland & Ohlsson 1978). Radioimmunoassays can measure proteins in concentrations down to $\mu\text{g/l}$. α_2 -M, α_1 -PI, ACHY, anti-thrombin III, α_2 -antiplasmin and IgA were quantified by electroimmunoassay (Laurell 1972). This is a rapid specific immunoprecipitation method. Less than 0.5 mg/l of the protein can rarely be detected.

It is not possible to measure proteases bound to α_2 -M with immunochemical methods. Therefore the synthetic chromogenic peptide substrate S-2484 was used to measure the granulocyte elastase bound to α_2 -M.

Qualitative analyses. The homogeneity of the different proteases and protease inhibitors was studied with regard to degradation products and protease/protease-inhibitor complexes by chromatographic separation on gel-filtration followed by immunoassay and crossed immunoelectrophoresis (Ganrot 1972, Grubb 1982). The different parts of the complexes could be identified with crossed immunoelectrophoresis using

intermediar gels containing antisera against the different proteases and the different protease inhibitors (Fig. 1).

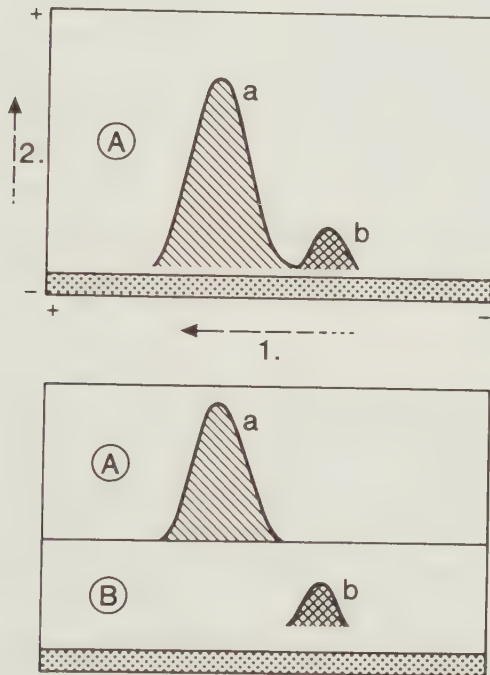


Fig 1. Crossed immunoelectrophoresis is performed in two steps. In the first, the antigens are separated by electrophoresis (1) and in the second, the separated antigens are forced into a gel containing antibodies (2) by the application of an electric field at right angles to the direction of the separation of the antigens in the first step.

Crossed immunoelectrophoresis of substance A using antisera against A results in precipitation pattern containing two peaks, a and b, with different electrophoretic mobility. Intermediar gel containing antisera against substance B precipitates peak b but not peak a. Thus, peak b represents complexes between substance A and B.

Immunohistochemistry. The unlabelled peroxidase antiperoxidase method (Sternberger et al 1970) with some modifications (Fryksmark et al 1982) was used for identification of the different pancreatic endoproteases and PSTI in specimens from metaplastic and normal gastro-intestinal mucosa. Unspecific staining is common in immunohistochemistry. It is therefore mandatory to test the specificity of the staining reaction by including different controls. The staining reaction was controlled by the use of the specific antisera after previous absorption with their appropriate antigens, and by the use of nonimmune serum instead of the specific antisera. Both methods gave complete blocking of the staining reactions. The basis for immunohistochemical studies is, of course, the specificity of the available antiserum to the protein studied.

RESULTS AND DISCUSSION

Determination and characterization of i.r. cationic trypsin, chymotrypsin and pancreatic cationic elastase in extracts from faeces and ileostomy drainage (paper I)

The extractable amounts of the different pancreatic endoproteases in faeces and ileostomy contents were measured with radioimmunoassay. The immunoreactivity of the different pancreatic endoproteases was identical in the DFP inactivated faecal samples, ileostomy contents and the purified DFP inactivated pancreatic endoproteases used as standards as shown by dilution experiments and gel-filtration studies. Thus, the enzymes retained their antigenic sites and did not change qualitatively during the passage through the intestinal canal. The levels of the different endoproteases in the two spots samples, taken from each stool, were comparable. This is in agreement with earlier findings (Haverback et al 1963, Ammann & Kashiwagi 1966).

After the passage through the small intestine roughly 50-200 mg/24 hours of the different extractable endoproteases studied reach the colon, according to the measurements on ileostomy contents. These values are similar to those found by others (Roy et al 1967) using enzymatic methods. During the passage through the colon, normally most of the extractable endo-

proteases were degraded and only trace amounts (<1 mg/ 24 hours) were found in faecal extracts from subjects not treated with antibiotics during the last two years. These trace amounts of i.r. pancreatic endoproteases correspond well to those found by others (Borgström et al 1977, Florholmen 1985). In subjects treated with antibiotics during the year preceding the study an increased level of trypsin, corresponding to roughly 70mg/24 hours was found, while the levels of chymotrypsin and elastase were about 1 mg/24 hours. Thus the impaired degradation of trypsin and elastase earlier found in subjects after recent or during ongoing antibiotic treatment (Borgström et al 1977) has, in this study, been shown to be long-lasting (more than 1 year) for trypsin, while the levels of chymotrypsin and elastase returned to low levels within a year.

Turnover of intraduodenally instilled radiolabelled trypsin in man (paper II)

Human cationic trypsin was labelled with ^{131}I by a lactoperoxidase method (Thorell & Johansson 1971). The labelled trypsin retained the substrate cleaving activity and was when incubated with serum bound to the plasma protease inhibitors in the same manner as active trypsin. Thus, it was demonstrated that all the labelled trypsin retained its biological activity.

After intraduodenal instillation of the active ^{131}I -labelled human cationic trypsin in healthy volunteers and subjects with ileostomy, 90% of the instilled radioactivity was recovered in 3-day collections of faeces, ileostomy contents and urine.

The fate of the labelled enzyme was studied by analysis of plasma, urine, faeces and ileostomy contents. The recovered radioactivity in ileostomy contents and faeces was characterized after centrifugation of faecal and ileostomy content homogenates. The radioactivity was found to be partly bound to particular substances (seen in the analysis of pellets) and partly extractable (i.e. found in supernatant fluids). The extractable labelled substances were further characterized by

dialyses and gel-filtration. The extractable radioactivity was found to be high-molecular-weight-bound (molecular weight > 10,000 daltons), oligo-peptide-bound or in the form of free ^{131}I (low-molecular-weight-bound).

In accordance with the results of others (Borgström et al 1957) it was found that the major part of the labelled trypsin was degraded during the passage through the small intestine, only about 20 % of the instilled radioactivity being recovered in ileostomy contents during the 72-hour period.

The extractable high-molecular-weight-bound radioactivity constituted 4% of the instilled dose in the ileostomy contents and 0.5% in faecal samples from subjects not treated with antibiotics the year preceding the study. Thus, the high-molecular-weight-bound radioactivity, probably representing undegraded trypsin, was degraded during the passage through the large bowel. One individual, treated with antibiotics shortly before the study, had a high-molecular-weight-bound radioactivity in faeces corresponding to 3.5% of the instilled dose, which is roughly the same amount as in ileostomy contents. This finding illustrates an impaired degradation of trypsin in the large bowel after antibiotic treatment (Borgström et al 1977, paper I). The low-molecular-weight-bound radioactivity was found, for the major part, to be peptide-bound, probably representing degraded trypsin, and for the minor part to be free iodine. The free iodine probably represented iodine secreted into the gastro-intestinal tract and not de-iodination of the labelled trypsin in intestinal contents, as direct incubation of duodenal juice and labelled trypsin only resulted in the production of labelled degradation products and no free iodine.

The radioactivity bound to particular substances, corresponded to 11% of the instilled dose in ileostomy contents and roughly the same in faecal samples. The particular-bound radioactivity probably represented active trypsin bound to desquamated cells as suggested by Goldberg et al (1968b). Trypsin bound to particular substances can also explain the difference in tryptic

activity in faecal homogenates found by others (Dürr et al 1978) compared with the small amounts of i.r. trypsin found in faecal supernatant fluids (Borgström et al 1977, Florholmen 1985, paper I).

During the 72-hour period after the intraduodenal administration of ^{131}I -trypsin no high-molecular-weight-bound radioactivity was present in plasma. On the contrary, all recovered radioactivity in plasma corresponded to free ^{131}I . This is in contrast to the results of recent studies (Heinrich et al 1979, Lake Bakaar et al 1980).

The de-iodinating mechanism acting during the passage from intestine to the bloodstream of ^{125}I -labelled peptides documented in suckling rats (Jones 1977) seems also to exist in man as judged from our characterization of low-molecular-weight radioactivity in plasma. This finding also demonstrates that it is necessary to give non-labelled iodine in gastrointestinal turnover studies with I-labelled proteins. This is necessary not only to block the uptake in the thyroid gland but also to prevent an overestimation of high-molecular-weight-bound radioactivity in plasma that only represents free iodine being bound to iodine-binding proteins (Skogh 1982). One might argue that trypsin might be absorbed after ^{131}I is split off from the enzyme. However, earlier investigations have demonstrated no increase in the plasma level of trypsin or trypsin in complex with $\alpha_1\text{-PI}$ after stimulation of the pancreatic gland in healthy individuals (Borgström & Ohlsson 1978).

Studies on possible pancreatic uptake and resecretion of trypsin from the blood in the dog (paper III)

An enteropancreatic recirculation of trypsin (Götze & Rothman 1975) requires that active trypsin is absorbed from the intestine and then carried to the pancreatic gland with the blood and is finally resecreted as trypsinogen. We decided to test the proposed mechanism by analysing the fate of active homologous trypsin after intravenous injection in dogs. We found that the trypsin was immediately bound to the plasma protease

inhibitors, mainly α_1 -PI and α -M, and was eliminated from the circulation with a half-life of 15 minutes. This is in agreement with earlier studies (Ohlsson 1971).

Samples of pancreatic juice were collected every 15 minutes. Only trace amounts of protein-bound radioactivity appeared. During 3 hours the level of anionic canine trypsin in pancreatic juice was stable, whereas the level of protein-bound radioactivity steadily decreased. The total protein-bound radioactivity recovered in a 3-hour collection of pancreatic juice was less than 0.2% of the injected radioactivity. These results are in agreement with the findings of others (Levitt et al 1981, Rohr et al 1981) and speak against a substantial enteropancreatic recirculation of pancreatic enzymes as suggested by others (Götze & Rothman 1975, Lake Bakaar et al 1980).

Immunohistochemical demonstration of pancreatic endoprotease like and PSTI-like immunoreactivity in normal and metaplastic gastrointestinal mucosa (papers IV&V)

It has been suggested that trypsin and chymotrypsin bind to the intestinal mucosa cells, and thereby retain their enzymatic activity (Goldberg et al 1969). In the present study, specimens from gastrointestinal mucosa of normal and metaplastic types were analysed with the unlabelled antibody enzyme method (PAP-method) (Sternberger et al 1970, Fryksmark et al 1982) for trypsin-like immunoreactivity (paper IV). A marked positive staining for trypsin-like immunoreactivity was seen in the Paneth cells. The Paneth cells were identified on the basis of histological criteria and their content of lysozyme (Sandow & Whitehead 1979).

Paneth cells are normally found in the base of the crypts of Lieberkühn in the small intestine (Sandow & Whitehead 1979) but may also be found in metaplastic areas in gastric and colonic mucosa (Lewin 1969). Paneth cells, which have a morphological resemblance to pancreatic acinar cells, are secretory epithelial cells and contain heavy metals, lysozyme, phospholipase A2, IgG, IgA which it is suggested that they

secrete into the gut lumen (Elmes 1976, Rodning et al 1976, Sandow & Whitehead 1979, Senegas-Balas et al 1984). The function of the Paneth cells is not clearly understood, but it has been suggested that they control the bacterial microflora of the small intestine (Sandow & Whitehead 1979).

The demonstration of trypsin-like immunoreactive material in the Paneth cells prompted us to further analyse gastro-intestinal mucosa for different immunoreactive pancreatic endoproteases and PSTI (paper V). In the first study (paper IV) the cationic trypsin antiserum used had a slight cross-reactivity against anionic trypsin. In order to further characterize the trypsin-like immunoreactivity, anionic trypsin was purified from pancreatic juice and a monospecific antiserum against anionic trypsin was produced. Cross-reacting antibodies in the two antisera were then eliminated by an immunoabsorption technique with the isolated antigens bound to a solid phase.

A trypsin-like immunoreactivity of both anionic and cationic types as well as PSTI-like immunoreactivity was found in the Paneth cells in both small intestinal mucosa and metaplastic gastric and colonic mucosa including cases with ulcerative colitis. No chymotrypsin or elastase-like immunoreactive material was identified in any of the specimens. PSTI immunoreactive material was, in addition to being found in the Paneth cells, also found in the normal gastric and colonic mucosa and in some goblet cells of the small intestine, while no pancreatic endoprotease-like material was found in these cells.

It has been found that the number of Paneth cells increases in patients with chronic pancreatitis (Senegas-Balas et al 1982). The Paneth cells and the goblet cells increase in size in hamsters after pancreatic duct ligation (Balas et al 1980). These results argue against an absorption of the trypsin and PSTI-like immunoreactive material in the Paneth cells.

The present studies have demonstrated the presence of both a protease (trypsin) and the appropriate protease inhibitor

(PSTI) in the same type of cells. These results fit the theories of Laskowski; that tissues containing protease inhibitor also contain the appropriate protease. Furthermore, the demonstrated PSTI-like immunoreactive material in the goblet cells could be the inhibitor of the recently demonstrated goblectin found in the goblet cells, as goblectin is a proteolytic enzyme of serine type with almost the same properties as trypsin (Nexö et al 1984).

Immunoreactive PSTI and cationic trypsin in sera from patients with ulcerative colitis (paper VI)

In patients with acute attacks of ulcerative colitis an increased serum level of i.r. PSTI was found, while the serum level of i.r. cationic trypsin was within normal limits. Thus, a leakage of these proteins from the pancreatic gland is not a likely explanation of the increased PSTI levels, as the ratio of PSTI to cationic trypsin is fairly constant even after stimulation of the pancreatic gland (Eddeland & Wehlin 1978). Increased serum levels of i.r. PSTI are reported in patients with non-pancreatic malignancy (Matsuda et al 1983), bronchopneumonia and gynaecological disease (Huhtala et al 1982, 1983, Murata et al 1983) and after major gastro-intestinal surgery (Matsuda et al 1985). These reports suggest an extrapancreatic source of PSTI as do our results in acute colitis. The recent demonstration of i.r. PSTI in gastro-intestinal mucosa (paper V) could be the extrapancreatic source of PSTI explaining the increased serum level found in patients with acute colitis.

Pancreatic and granulocytic serine proteases in faecal extracts from patients with acute ulcerative colitis (paper VII)

In order to study the proteolytic process involved in the destruction of the colonic mucosa in patients with acute colitis, stool samples, consisting of a mixture of faeces, pus, desquamated cells and exudate from the colonic mucosa, were analysed regarding the protease/protease-inhibitor balance.

A marked proteolytic activity was demonstrated in supernatant fluids of faecal extracts from patients with acute severe

ulcerative colitis. In contrast no proteolytic activity was found in the supernatant fluid from faecal extracts from patients with quiescent disease or from healthy subjects with no history of recent antibiotic treatment. After the addition of DFP, a serine protease inhibitor, all proteolytic activity was inhibited in the different samples. Native α_1 -PI, one of the major plasma protease inhibitors, inhibited all proteolytic activity by forming complexes with the active proteases when added to the faecal extracts. Proteases in complex with α_1 -PI have an increased anionic electrophoretic mobility compared with the native protease. This fact made it possible to determine whether the recovered proteases were active or not by comparing the precipitation pattern from crossed immunoelectrophoresis before and after the addition of native α_1 -PI to the different samples. Five different active serine proteases were demonstrated by this method; two granulocytic proteases; elastase and neutral protease and three pancreatic endoproteases; anionic trypsin, anionic and cationic elastase. The method was only used qualitatively and not quantitatively.

Anionic trypsin, cationic trypsin, chymotrypsin, pancreatic cationic elastase and granulocyte elastase were therefore also quantified by radioimmunoassay in faecal samples. In subjects with acute colitis an increased level of i.r. granulocyte elastase was found, as compared with healthy subjects, demonstrating that the granulocytes released at least a part of their protease content. This finding is supported by studies with ^{111}In -labelled granulocytes in patients with acute attacks of ulcerative colitis (Saverymuttu et al 1984, 1985). Labelled granulocytes have been demonstrated in the colonic wall by scanning and during a 4-day period half of the injected labelled granulocytes were recovered in faeces (Saverymuttu et al 1984, 1985).

Immunoreactive pancreatic endoproteases in faecal samples from healthy subjects with no history of recent antibiotic treatment are only found in trace amounts (Borgström et al 1977, paper I). In faecal samples from patients with acute

ulcerative colitis an increased levels of cationic elastase and anionic trypsin were found while the levels of chymotrypsin and cationic trypsin were the same as in controls. These findings indicate an altered degradation of elastase and anionic trypsin in patients with acute colitis. It is probably the colonic degradation of these enzymes that is altered since in normal ileostomy contents the level of cationic trypsin is about twice that of chymotrypsin and elastase (paper I). Quantitation with radioimmunoassay showed that granulocytic elastase was present only as a trace protein in normal ileostomy contents.

Protease inhibitors in plasma and faecal extracts from patients with acute ulcerative colitis (paper VIII)

The level and functional activity of the major protease inhibitors in plasma and faecal extracts were analysed in a consecutive series of patients admitted with their first attack of acute, severe ulcerative colitis. The patients were retrospectively divided into two groups; one with total colitis, and another with distal colitis.

The protease-inhibiting capacity in faecal extracts was saturated. After chromatographic separation of faecal extracts α_2 -M, α_1 -PI and ACHY eluted in volumes corresponding to the native proteins. In some samples a small additional peak of α_1 -PI was found in a volume corresponding to protease- α_1 -PI complexes. Characterization of immunoreactive α_1 -PI with crossed immunoelectrophoresis showed only one peak in the majority of samples. Electrophoretically this peak had a decreased anionic mobility compared with native α_1 -PI. No change in the crossed immunoprecipitation pattern of α_1 -PI in faecal extracts was obtained after adding increasing amounts of trypsin to the faecal extracts. Thus, α_1 -PI in faeces was inactive regarding the protease-inhibiting function. An inactive α_1 -PI in an uncomplexed form with a slightly reduced anionic mobility has also been found in sputum from patients with acute bronchitis (Ohlsson & Tegner 1975, Stockley et al 1982), and probably represents the modified inhibitor part of a dissociated protease- α_1 -PI

complex (Tegner 1978). Some samples contained two additional peaks on crossed immunoelectrophoresis. These peaks were characterized as α_1 -PI in complex with the granulocyte proteases elastase and neutral protease.

ACHY in faecal extracts was also functionally inactive. Characterization of ACHY in faecal extracts with crossed immunoelectrophoresis before and after the addition of chymotrypsin showed only one peak. In some samples this peak migrated more slowly than the free native ACHY.

α_2 -M was without residual inhibiting capacity. It is difficult to measure and characterize proteases bound to α_2 -M with immunochemical techniques. However, small peptides acting as specific substrates can reach and be cleaved by the protease captured within the inhibitor molecules. With methods based on this property of the complex we could demonstrate a granulocyte elastase-like enzymatic activity bound to α_2 -M.

It was also found in the present study that the plasma level of α_2 -M was decreased in patients with total colitis and normal in patients with distal disease. This finding is in agreement with other studies (Brown et al 1980). The plasma levels of α_1 -PI and ACHY were increased with no difference between the two groups of patients. α_2 -M is one of the major plasma protease inhibitors with a capacity to inhibit all serine proteases including pancreatic and granulocytic proteases as well as plasmin and thrombin. The plasma level of α_2 -M is within the normal range in most diseases but in sepsis and pancreatitis low serum levels have been reported (Gallimore et al 1980, McMahon et al 1984, Lasson & Ohlsson 1984). These diseases are characterized by the liberation of great amounts of proteolytic enzymes, which probably explains the decreased α_2 -M levels seen, as the protease- α_2 -M complexes are rapidly eliminated and degraded - inhibitor as well as protease (Ohlsson 1971, Balldin et al. 1978, Ohlsson et al 1982). A similar mechanism might exist in acute colitis which, according to the present studies, is characterized by a pronounced protease release. Besides a free proteolytic acti-

vity, complexes between granulocyte proteases and α_2 -M and α_1 -PI were found in the faecal extracts from patients with acute ulcerative colitis. Activation of the different cascade systems, such as the fibrinolytic and coagulation systems, could also lead to the release of proteases and the consumption of inhibitors. The major rapid inhibitors of plasmin and thrombin are α_2 -antiplasmin and antithrombin III (Collen 1980, Williams 1983). In this study the plasma levels of these inhibitors were slightly increased, demonstrated by both immunological and enzymatical methods, indicating free and uncomplexed inhibitors in these systems. These results argue against any major consumption of α_2 -M due to plasmin or thrombin release.

The plasma level of IgM was within normal limits as has been earlier reported in patients with quiescent or acute attacks of ulcerative colitis (Weeke & Jarnum 1971, Bendixen et al 1970). Haemodilution or increased losses of macromolecules through the intestinal wall thus seem a less likely explanation of the low α_2 -M plasma level found in patients with total colitis.

Our demonstration of complex formation of α_2 -M and α_1 -PI with the granulocytic proteases, elastase and neutral protease, and the recent demonstration of increased plasma levels of granulocyte elastase in complex with α_1 -PI in patients with acute ulcerative colitis (Adeyemi et al 1985) suggest that the decreased plasma level of α_2 -M in patients with acute ulcerative colitis is due to the release of granulocytic proteases followed by complex formation and consumption of the major plasma inhibitor α_2 -M. A consumption of α_1 -PI and ACHY is less readily demonstrated in plasma as these protease inhibitors also react as acute-phase proteins.

CONCLUDING REMARKS AND CLINICAL IMPLICATIONS

The major part of the proteolytic enzymes from pancreatic juice is degraded during the passage through the small intestine. A minor part is already bound to particular substances in the small intestine and passes through the colon without further degradation. The unbound proteases reaching the caecum are normally degraded by the bacterial flora in the colon. Antibiotic treatment impairs this degradation and the effect is long-lasting, at least for trypsin. The clinical implication of this finding is not clear yet. However, it might contribute to some of the adverse effects observed after antibiotic treatment.

In the present studies no evidence of a physiological significant enteropancreatic recirculation of active pancreatic endoproteases was demonstrated. Even if some active proteases are absorbed from the intestinal contents, they are immediately bound to the major serum protease inhibitors and eliminated from the circulation and degraded mainly in the liver, and are not secreted in pancreatic juice.

After intraduodenal administration of ^{131}I -labelled trypsin only free ^{131}I was found in plasma samples. Obviously a de-iodinating mechanism exists in the small intestinal tract. Therefore, if gastro-intestinal turnover studies with I-labelled substances are performed, the recovered radioactivity must be carefully characterized. If iodine-binding sites of plasma proteins are not blocked by pretreatment with nonlabelled iodine, an overestimation of absorbed macromolecules will result.

The Paneth cells in the gastro-intestinal mucosa contain anionic and cationic trypsin-like and PSTI-like immunoreactive material, but no chymotrypsin and elastase-like immunoreactive material. The findings of both immunoreactive anionic and cationic trypsin as well as their appropriate inhibitor, PSTI, in the Paneth cells, demonstrate a probable extrapancreatic source of these proteins, and are a further indication of the

resemblance between the Paneth cells and the acinar pancreatic cells. In addition to Paneth cells, the normal gastric and colonic mucosa and some goblet cells in the small intestine contain PSTII-like material. These cells do not contain pancreatic endoprotease-like immunoreactivity.

Patients with acute ulcerative colitis have an increased serum level of i.r.PSTII while the serum level of i.r.cationic trypsin is within the normal range. These findings suggest an additional extrapancreatic source of PSTII. The gastrointestinal mucosa might be one.

Supernatant fluids from faecal extracts from patients with acute colitis contain material from faeces, pus and exudates from the inflamed colonic wall. The protease/protease-inhibitor balance in these fluids, thus probably reflects the situation in the colonic mucosa in patients with acute colitis. Faecal supernatant fluids from patients with acute colitis contain a marked proteolytic activity. Active proteolytic enzymes of both granulocytic and pancreatic origins are detected.

The extractable protease-inhibitors in stools from patients with acute colitis are, for the major part, present in an inactive form, unable to bind proteases, either representing the inhibitor part of a dissociated protease/protease-inhibitor complex or inhibitor molecules inactivated by other proteolytic processes or by oxidation. A minor part of α_1 -PI and α_2 -M is present in complex with the granulocytic proteases elastase and neutral protease.

Extension of the disease is well reflected by the plasma level of α_2 -M. A decreased plasma level of α_2 -M indicates total colitis and may be useful in determining the extent of the disease during the first day of illness. The decreased plasma level of α_2 -M is due to complex formation with granulocytic proteases followed by elimination via the RES system and hepatocytes.

The presence of active proteases in the faecal extracts from patients with acute ulcerative colitis probably reflects a local protease/protease-inhibitor imbalance in the damaged mucosa-submucosa. Local treatment with an appropriate protease inhibitor in addition to conventional therapy thus seems to be worthwhile as a therapeutic step to reduce tissue damage.

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References

- Abita JP, Delaage M, Lazdunski M. 1969 The mechanism of activation of trypsinogen. *Eur J Biochem* 8:314-324
- Adeyemi EO, Neumann S, Chadwick VS, Hodgson HJF, Pepys MB. 1985 Circulating human leucocyte elastase in patients with inflammatory bowel disease. *Gut* 26:1306-1311
- Ammann R, Kashiwagi H. 1966 Pancreatic exocrine insufficiency and proteolytic enzymes in stool. *Helv Med Acta* 33:220-228
- Aronsen K-F, Ekelund G, Kindmark C-O, Laurell C-B. 1972 Sequential changes of plasma proteins after surgical trauma. *Scand J Clin Lab Invest* 29, suppl 124:127-136
- Balas D, Senegas-Balas F, Bertrand C, Frexinos J, Ribet A. 1980 Effects of pancreatic duct ligation on the hamster intestinal mucosa. *Digestion* 20:157-167
- Balldin G, Laurell C-B, Ohlsson K. 1978 Increased catabolism of α -macroglobulins after intravenous infusion of trypsin- α_1 -antitrypsin complexes in dogs. *Hoppe-Seyler's Z Physiol Chem* 359:699-708
- Barrett AJ, Starkey PM. 1973 The interaction of α_2 -macroglobulin with proteinases. *Biochem J* 133:709-724
- Baugh RJ, Travis J. 1976 Human leukocyte granule elastase: Rapid isolation and characterization. *Biochemistry* 15:836-841
- Beatty K, Travis J, Bieth J. 1982 The effect of α_2 -macroglobulin on the interaction of α_1 -proteinase inhibitor with porcine trypsin. *Biochim Biophys Acta* 704:221-226
- Bendixen G, Goltermann N, Jarnum S, Jensen KB, Weeke B, Westergaard H. 1970 Immunoglobulin and albumin turnover in ulcerative colitis. *Scand J Gastroent* 5:433-441
- Bergstrand LO, Gustafsson BE, Holmström B, Norin KE. 1981 The physiological activity of the intestinal flora in patients with Crohn's disease studied by inoculation of germfree rats. *Acta Chir Scand* 147:711-715
- Bernier JJ, Desmazures CH, Florent CH, Aymes CH, L'Hirondel CH. 1978 Diagnosis of protein-losing enteropathy by gastrointestinal clearance of α_1 -antitrypsin. *Lancet* ii:763-764
- Bicks RO, Kirsner JB, Palmer WL. 1959 Serum proteins in ulcerative colitis. I Electrophoretic patterns in active disease. *Gastroenterology* 37:256-262
- Borgström A. 1979 Studies on the endogenous turnover of pancreatic endoproteases with special reference to trypsin in man and dog. Thesis, University of Lund

- Borgström A, Ohlsson K. 1976 Radioimmunological determination and characterization of cathodal trypsin-like immunoreactivity in normal human plasma. *Scand J Clin Lab Invest* 36:809-814
- Borgström A, Ohlsson K. 1978 Studies on the turnover of endogenous cathodal trypsinogen in man. *Eur J Clin Invest* 8:379-382
- Borgström A, Genell S, Ohlsson K. 1977 Elevated fecal levels of endogenous pancreatic endopeptidases after antibiotic treatment. *Scand J Gastroenterol* 12:525-529
- Borgström A, Kukora J, Ohlsson K. 1980 Studies on immunoreactive pancreatic elastase 2 in human serum. *Hoppe-Seyler's Z Physiol Chem* 361:633-640
- Borgström B, Dahlqvist A, Lundh G, Sjövall J. 1957 Studies of intestinal digestion and absorption in the human. *J Clin Invest* 36:1521-1536
- Boudier C, Holle C, Bieth JG. 1981 Stimulation of the elastolytic activity of leukocyte elastase by leukocyte cathepsin G. *J Biol Chem* 256:10256-10258
- Bounous G. 1982 Acute necrosis of the intestinal mucosa. *Gastroenterology* 82:1457-1467
- Bounous G, Brown RA, Mulder DS, Hampson LG, Gurd FN. 1965 Abolition of 'tryptic enteritis' in the shocked dog. *Arch Surg* 91:371-375
- Bounous G, McArdle AH, Hodges DM, Hampson LG, Gurd FN. 1966 Biosynthesis of intestinal mucin in shock: relationship to tryptic hemorrhagic enteritis and permeability to curare. *Ann Surg* 164:13-22
- Bounous G, Menard D, de Medicis E. 1977 Role of pancreatic proteases in the pathogenesis of ischemic enteropathy. *Gastroenterology* 73:102-108
- Bounous G, Proulx J, Konok G, Wollin A. 1979 The role of bile and pancreatic proteases in the pathogenesis of ischemic enteropathy. *Int J Clin Pharm Biopharm* 17:317-323
- Brown DJC, Khan JA, Copeland G, Jewell DP. 1980 Alpha₂-macroglobulin in patients with inflammatory bowel disease. *J Clin Lab Immunol* 4:53-57
- Cassiman JJ, van Leuven F, van der Schueren B, van den Berghe H. 1980 Immunohistochemical localization of human α_2 -macroglobulin in connective tissue. *Cell Tissue Res* 213:301-310
- Collen D. 1980 On the regulation and control of fibrinolysis. *Thromb Hemost* 43:77-89

- Crossley JR, Elliott RB. 1977 Simple method for diagnosing protein-losing enteropathies. *Br Med J* 1:428-429
- Davidson O, Christensen EI, Gliemann J. 1985 The plasma clearance of human α_2 -macroglobulin-trypsin complex in the rat is mainly accounted for by uptake into hepatocytes. *Biochim Biophys Acta* 846:85-92
- Dickson I, Alper CA. 1974 Changes in serum proteinase inhibitor levels following bone surgery. *Clin Chim Acta* 54:381-385
- de Dombal FT. 1968 Prognostic value of the serum proteins during severe attacks of ulcerative colitis. *Gut* 9:144-149
- Dürr HK, Schneider R, Bode C, Bode JC. 1978 Fecal chymotrypsin: Study on some characteristics of the enzyme. *Digestion* 17:396-403
- Eddeland A, Ohlsson K. 1978a Purification and immunochemical quantitation of human pancreatic secretory trypsin inhibitor. *Scand J Clin Lab Invest* 38:261-267
- Eddeland A, Ohlsson K. 1978b A radioimmunoassay for measurement of human pancreatic secretory trypsin inhibitor in different body fluids. *Hoppe-Seyler's Z Physiol Chem* 359:671-675
- Eddeland A, Wehlin L. 1978 Secretin/cholecystokinin-stimulated secretion of trypsinogen and trypsin inhibitor in pure human pancreatic juice collected by endoscopic retrograde catheterization. *Hoppe-Seyler's Z Physiol Chem* 359:1653-1658
- Edwards FC, Truelove SC. 1963 The course and prognosis of ulcerative colitis. *Gut* 4:299-315
- Edwards FC, Truelove SC. 1964 The course and prognosis of ulcerative colitis. Part III Complications. *Gut* 5:1-22
- Ekerot L, Ohlsson K. 1982 The elimination of α_2 -macroglobulin complexes from the arthritic joint. An experimental study in dogs. *Scand J Plast Reconstr Surg* 16:107-115
- Ekerot L, Ohlsson K, Rank F. 1982 The elimination of α_2 -macroglobulin complexes from the arthritic joint. A clinical study in rheumatoid arthritis. *Scand J Plast Reconstr Surg* 16:295-299
- Elmes ME. 1976 The Paneth cell population of the small intestine of the rat - effects of fasting and zinc deficiency on total count and on dithizone-reactive count. *J Path* 118:183-191

- Feinstein G, Janoff A. 1975 A rapid method for purification of human granulocyte cationic neutral proteases: Purification and characterization of human granulocyte chymotrypsin-like enzyme. *Biochim Biophys Acta* 403:477-492
- Feinstein G, Hofstein R, Koifmann J, Sokolovsky M. 1974. Human pancreatic proteolytic enzymes and protein inhibitors. *Eur J Biochem* 43:569-581
- Florholmen J. 1985 A study on human and porcine cationic trypsin-like immunoreactivity. Thesis, University of Tromsø, Norway
- Fryksmark U, Ohlsson K, Polling Å, Tegner H. 1982 Distribution of antileukoprotease in upper respiratory mucosa. *Ann Otol Rhinol Laryngol* 91:268-271
- Gallimore MJ, Aasen AO, Smith Erichsen N, Larsbraaten M, Lyngaas K, Amundsen E. 1980 Plasminogen concentrations and functional activities and concentrations of plasmin inhibitors in plasma samples from normal subjects and patients with septic shock. *Thromb Res* 18:601-608
- Ganrot PO. 1972 Crossed immunoelectrophoresis. *Scand J Clin Lab Invest* 29 suppl 124:39-47
- Genell S, Gustafsson BE. 1977 Impaired enteric degradation of pancreatic endopeptidases in antibiotic-treated rats. *Scand J Gastroent* 12:801-809
- Genell S, Gustafsson BE, Ohlsson K. 1976 Quantitation of active pancreatic endopeptidases in the intestinal contents of germfree and conventional rats. *Scand J Gastroent* 11:757-762
- Genell S, Gustafsson BE, Ohlsson K. 1977 Immunochemical quantitation of pancreatic endopeptidases in the intestinal contents of germfree and conventional rats. *Scand J Gastroent* 12:811-820
- Geokas M, Largman C, Brodrick J, Johnson J, Fasett M. 1979 Immunoreactive forms of human pancreatic chymotrypsin in normal plasma. *J Biol Chem* 254:2775-2781
- Goldberg DM, Campbell R, Roy AD. 1968a Binding of trypsin and chymotrypsin by human intestinal mucosa. *Biochim Biophys Acta* 167:613-615
- Goldberg DM, McAllister RA, Roy AD. 1968b Studies on human intestinal proteolysis. *Scand J Gastroent* 3:193-201
- Goldberg DM, Campbell R, Roy AD. 1969 Studies on the binding of trypsin and chymotrypsin by human intestinal mucosa. *Scand J Gastroent* 4:217-226

- Goligher JC, Hoffman DC, de Dombal FT. 1970 Surgical treatment of severe attacks of ulcerative colitis, with special reference to the advantages of early operation. *Br Med J* 4:703-706
- Greene LJ, Rigbi M, Fackre DS. 1966 A trypsin inhibitor from bovine pancreatic juice. *J Biol Chem* 241:5610-5618
- Grubb AO. 1983 Crossed immunoelectrophoresis. *Scand J Immunol* 10:113-124
- Guy O, Lombardo D, Bartelt DC, Amic J, Figarella C. 1978 Two human trypsinogens. Purification, molecular properties and N-terminal sequences. *Biochemistry* 17:1669-1675
- Götze H, Rothman SS. 1975 Enteropancreatic circulation of digestive enzyme as a conservation mechanism. *Nature* 257:607-609
- Haverback BJ, Dyce BJ, Gutentag J, Montgomery DW. 1963 Measurement of trypsin and chymotrypsin in stool. *Gastroenterology* 44:588-597
- Heinrich HC, Gabbe EE, Brüggemann J, Icagic F. 1979 Enteropancreatic circulation of trypsin in man. *Klin Wschr* 57:1295-1297
- Huhtala M-L, Pesonen K, Kalkkinen N, Stenman U-H. 1982. Purification and characterization of a tumor-associated trypsin inhibitor from the urine of a patient with ovarian cancer. *J Biol Chem* 257:13713-13716
- Huhtala M-L, Kahanpää K, Seppälä M, Halila H, Stenman U-H. 1983 Excretion of a tumor-associated trypsin inhibitor (TATI) in urine of patients with gynecological malignancy. *Int J Cancer* 31:711-714
- Johnson GG, Morris JM, Berk RS. 1967 The extracellular protease from *Pseudomonas aeruginosa* exhibiting elastase activity. *Can J Microbiol* 13:711-719
- Johnson U, Ohlsson K, Olsson I. 1976 Effects of granulocyte neutral proteases on complement components. *Scand J Immunol* 5:421-426
- Jones RE. 1977 De-iodination of labelled protein during intestinal transmission in the suckling rat. *Proc R Soc Lond B* 199:279-290
- Karbach U, Ewe K, Bodenstern H. 1983 Alpha₁-antitrypsin, a reliable endogenous marker for intestinal protein loss and its application in patients with Crohn's disease. *Gut* 24:718-723
- Kazal LA, Spicer DS, Brahinsky RA. 1948 Isolation of a crystalline trypsin inhibitor-anticoagulant protein from pancreas. *J Am Chem Soc* 70:3034-3040

- Kirsner JB, Shorter RG. 1982a Recent developments in "non-specific" inflammatory bowel disease (First of two parts). *N Engl J Med* 306:775-785
- Kirsner JB, Shorter RG. 1982b Recent developments in non-specific inflammatory bowel disease (Second of two parts). *N Engl Med* 306:837-848
- Kukral JC, Adams AP, Preston FW. 1965 Protein producing capacity of the human exocrine pancreas: Incorporation of S^{35} methionine in serum and pancreatic juice protein. *Ann Surg* 162:63-73
- Lake-Bakaar G, Rubio CE, McKavanagh S, Potter BJ, Summerfield JA. 1980 Metabolism of I^{125} -labelled trypsin in man: evidence for recirculation. *Gut* 21:580-586
- Largman C, Brodrick JW, Geokas MC. 1976 Purification and characterization of two human pancreatic elastases. *Biochemistry* 15:2491-2500
- Largman C, Brodrick JW, Geokas MC, Johnson JH. 1978 Demonstration of human pancreatic anionic trypsinogen in normal serum by radioimmunoassay. *Biochim Biophys Acta* 543:450-454
- Lasson Å, Ohlsson K. 1984 Protease inhibitors in acute human pancreatitis. Correlation between biochemical changes and clinical course. *Scand J Gastroent* 19:779-786
- Laurell C-B. 1972 Electroimmunoassay. *Scand J Clin Lab Invest* 29, suppl 124:21-37
- Laurell C-B, Jeppson J-O. 1975 Protease inhibitors in plasma. In: *The Plasma Proteins*. Putnam FW (ed), Academic Press New York, pp 229-264
- Lazarus GS, Daniels JR, Brown RS, Bladen HA, Fullmer HM. 1968 Degradation of collagen by a human granulocyte collagenolytic system. *J Clin Invest* 47:2622-2629
- Lewin K. 1969 The Paneth cell in disease. *Gut* 10:804-811
- Levitt MD, Ellis CJ, Murphy SM, Schwartz ML. 1981 Study of the possible enteropancreatic circulation of pancreatic amylase in the dog. *Am J Physiol* 241:G54-G58
- McMahon MJ, Bowen M, Mayer AD, Cooper EH. 1984 Relation of α_2 -macroglobulin and other antiproteases to the clinical features of acute pancreatitis. *Am J Surg* 147:164-170
- Malawer SJ, Powell DW. 1967 An improved turbidimetric analysis of polyethylene glycol utilizing an emulsifier. *Gastroenterology* 53:250-256
- Mallory PA, Travis J. 1974 Human pancreatic enzymes: Purification and characterization of a nonelastolytic enzyme, protease E, resembling elastase. *Biochemistry* 14:722-730

- Marks WH, Ohlsson K. 1983 Elimination of pancreatic secretory trypsin inhibitor from the circulation. *Scand J Gastroenterol* 18:955-959
- Marner I-L, Friberg S, Simonsen E. 1975 Disease activity and serum proteins in ulcerative colitis. *Immunochemical quantitation. Scand J Gastroent* 10:537-544
- Matsuda K, Ogawa M, Murata A, Kitahara T, Kosaki G. 1983 Elevation of serum immunoreactive pancreatic secretory trypsin inhibitor contents in various malignant diseases. *Res Comm Chem Path Pharm* 40:301-305
- Matsuda K, Ogawa M, Shibata T, Nishibe S, Miyauchi K, Matsuda Y, Mori T. 1985 Postoperative elevation of serum pancreatic secretory trypsin inhibitor. *Am J Gastroent* 80:694-698
- van de Merwe JP, Mol GJJ. 1982 Levels of trypsin and α -chymotrypsin in feces from patients with Crohn's disease. *Digestion* 24:1-4
- Meyers S, Wolke A, Field SP, Fewer EJ, Johnson JW, Janowitz HD. 1985 Fecal α_1 -antitrypsin measurement: An indicator of Crohn's disease activity. *Gastroenterology* 89:1313-1318
- Morgenstern L, Hiatt N. 1967 Injurious effect of pancreatic secretions on postradiation enteropathy. *Gastroenterology* 53:923-928
- Morgenstern L, Patin CS, Krohn HL, Hiatt N. 1970 Prolongation of survival in lethally irradiated dogs. *Arch Surg* 101:586-589
- Morson BC, Dawson IMP. 1979 Ulcerative colitis. In: *Gastrointestinal Pathology*. Blackwell Scientific Publications, London, pp 523-561
- Murata A, Ogawa M, Matsuda K, Matsuura N, Kosaki G. 1983 Immunoreactive pancreatic secretory trypsin inhibitor in gynecological diseases. *Res Comm Chem Path Pharm* 41:493-499
- Murphy G, Reynolds JJ, Bretz U, Baggiolini M. 1977 Collagenase is a component of the specific granules of human neutrophil leucocytes. *Biochem J* 162:195-197
- Nexö E, Poulsen SS, Hansen SN, Kirkegaard P, Olsen PS. 1984 Characterisation of a novel proteolytic enzyme localised to goblet cells in rat and man. *Gut* 25:656-664
- Ohlsson K. 1971a Interaction in vitro and in vivo between dog trypsin and dog plasma protease inhibitors. *Scand J Clin Lab Invest* 28:219-223

- Ohlsson K. 1971b Elimination of ^{125}I -trypsin α -macroglobulin complexes from blood by reticuloendothelial cells in dog. *Acta Physiol Scand* 81:269-277
- Ohlsson K. 1974 Interaction between endogenous proteases and plasma protease inhibitors in vitro and in vivo. In: Bayer Symposium V "Proteinase Inhibitors". Fritz H, Tschesche H, Greene LJ, Truscheit E (eds) pp 96-105 Springer Verlag Berlin, Heidelberg, New York
- Ohlsson K, 1976 Collagenase and elastase released during peritonitis are complexed by plasma protease inhibitors. *Surgery* 79:652-657
- Ohlsson K. 1985 Granulocyte proteases and their inhibitors in inflammation; basic concepts and clinical implications. In: Inflammation. Basic mechanics, tissue injuring principles, and clinical models. Venge P, Lindbom A (eds), Almquist & Wiksell International, Stockholm, Sweden pp 379-393
- Ohlsson K, Laurell C-B. 1976 The disappearance of enzyme-inhibitor complexes from the circulation of man. *Clin Sci Mol Med* 51:87-92
- Ohlsson K, Olsson I. 1973 The neutral proteases of human granulocytes. Isolation and partial characterization of two granulocyte collagenases. *Eur J Biochem* 36:473-481
- Ohlsson K, Olsson I. 1974 Neutral proteases of human granulocytes. III Interaction between human granulocyte elastase and plasma protease inhibitors. *Scand J Clin Lab Invest* 34:349-355
- Ohlsson K, Olsson A-S. 1976 Purification and partial characterization of human pancreatic elastase. *Hoppe-Seyler's Z Physiol Chem* 357:1153-1161
- Ohlsson K, Olsson I. 1977a The extracellular release of granulocyte collagenase and elastase during phagocytosis and inflammatory processes. *Scand J Haematol* 19:145-152
- Ohlsson K, Olsson I. 1977b Neutral proteases of human granulocytes. IV Interaction between human granulocyte collagenase and plasma protease inhibitors. *J Lab Clin Med* 89:269-277
- Ohlsson K, Olsson A-S. 1978 Immunoreactive granulocyte elastase in human serum. *Hoppe-Seyler's Z Physiol Chem* 359: 1531-1539
- Ohlsson K, Tegner H. 1975 Granulocyte collagenase, elastase and plasma protease inhibitors in purulent sputum. *Eur J Clin Invest* 5:221-227
- Ohlsson K, Åkesson U. 1976 Alpha_1 -antichymotrypsin interaction with cationic proteins from granulocytes. *Clin Chim Acta* 73:285-291

- Ohlsson K, Ganrot P-O, Laurell C-B. 1971 In vivo interaction between trypsin and some plasma proteins in relation to tolerance to intravenous infusion of trypsin in dog. *Acta Chir Scand* 137:113-121
- Ohlsson K, Olsson I, Spitznagel J. 1977 Localization of chymotrypsin-like cationic protein, collagenase and elastase in azurophil granules of human neutrophilic polymorphonuclear leukocytes. *Hoppe-Seyler's Z Physiol Chem* 358:361-366
- Ohlsson K, Polling A, Stenberg A. 1982 Glutaminyl-peptide γ -glutamyltransferase dependence of the uptake of trypsin- α -macroglobulin complexes by macrophages. *Hoppe-Seyler's Z Physiol Chem* 363:213-220
- Otsuki M, Oka T, Suehiro I, Okabayashi Y, Ohki A, Yuu H, Baba S. 1984 Serum pancreatic secretory trypsin inhibitor in pancreatic disease. *Clin Chim Acta* 142:231-240
- Pacaud M. 1976 Purification of protease II from *Escherichia coli* by affinity chromatography and separation of two enzyme species from cells harvested at late log phase. *Eur J Biochem* 64:199-204
- Pacaud M, Richaud C. 1975 Protease II from *Escherichia coli*. Purification and characterization. *J Biol Chem* 250:7771-7779
- Pacaud M, Sibilli L, le Bras G. 1976 Protease I from *Escherichia coli*. Some physicochemical properties and substrate specificity. *Eur J Biochem* 69:141-151
- Parks DA, Granger DN, Bulkley GB, Shah AK. 1985. Soybean trypsin inhibitor attenuates ischemic injury to the feline small intestine. *Gastroenterology* 89:6-12
- Rodning CB, Wilson ID, Erlandsen SL. 1976 Immunoglobulins within human small-intestinal Paneth cells. *Lancet* i:984-987
- Rohr G, Kern H, Scheele G. 1981 Enteropancreatic circulation of digestive enzymes does not exist in the rat. *Nature* 292:470-472
- Roy AD, Campbell R, Biol LI, Goldberg M. 1967 Effect of diet on the trypsin and chymotrypsin output in the stools of patients with an ileostomy. *Gastroenterology* 53:584-589
- Ryley HC, Brogan ID. 1973 Quantitative immunoelectrophoretic analysis of the plasma proteins in the sol phase of sputum from patients with chronic bronchitis. *J Clin Pathol* 26:852-856
- Salvesen GS, Barrett AJ. 1980 Covalent binding of proteinases in their reaction with α_2 -macroglobulin. *Biochem J* 187:695-701

- Sandow MJ, Whitehead R. 1979 Progress report. The Paneth cell. Gut 20:420-431
- Savermuttu SH, Hodgson HJF, Chadwick VS. 1984 Comparison of fecal granulocyte excretion in ulcerative colitis and Crohn's colitis. Dig Dis Sci 29:1000-1004
- Savermuttu SH, Peters AM, Crofton MF, Rees H, Lavender JP, Hodgson HJF, Chadwick VS. 1985 ¹¹¹Indium autologous granulocytes in the detection of inflammatory bowel disease. Gut 26:955-960
- Schweitz H, Vincent JP, Lazdunski M. 1973 Trypsin-pancreatic secretory inhibitor (Kazal inhibitor) interaction. Kinetic and thermodynamic properties. Biochemistry 12:2841-2846
- Senegas-Balas F, Bastie MJ, Balas D, Escourrou J, Bommelaer G, Bertrand C, Arany Y, Ribet A. 1982 Histological variations of the duodenal mucosa in chronic human pancreatitis. Dig Dis Sci 27:917-922
- Senegas-Balas F, Balas D, Verger R, de Caro A, Figarella C, Ferrato F, Lechene P, Bertrand C, Ribet A. 1984 Immunohistochemical localization of intestinal phospholipase A2 in rat paneth cells. Histochemistry 81:581-584
- Shotton DM. 1970 Elastase. In: Methods in Enzymology, vol XIX Proteolytic Enzymes. Perlmann GE, Lorand L (eds) pp 113-139 Academic Press New York and London
- Skogh T. 1982 Overestimate of ¹²⁵I-protein uptake from the adult mouse gut. Gut 23:1077-1080
- Sternberger LA, Hardy PH Jr, Cuculis JJ, Meyer HG. 1970 The unlabelled antibody enzyme method of immunochemistry. Preparations and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. J Histochem Cytochem 18:315-333
- Stockley RA, Afford SC, Burnett D. 1982 The electrophoretic mobility of α_1 -proteinase inhibitor: effects of proteolysis and cigarette smoke. Hoppe-Seyler's Z Physiol Chem 363:387-393
- Tegner H. 1978 Quantitation of human granulocyte protease inhibitors in non-purulent bronchial lavage fluids. Acta Otolaryngol 85:282-289
- Thorell JI, Johansson BG. 1971 Enzymatic iodination of polypeptides with ¹²⁵I to high specific activity. Biochim Biophys Acta 251:363-369
- Truelove SC, Witts LJ. 1955 Cortisone in ulcerative colitis. Final report on a therapeutic trial. Br Med J 2:1041-1048

- Venge P, Olsson I. 1975 Cationic proteins of human granulocytes. Effects on the complement system and mediation of chemotactic activity. *J Immunol* 115:1505-1508
- Walker BE, Kelleher J, Davies T, Losowsky MS. 1971 Chemical faecal fat using single stools. *Scand J Gastroent* 6:277-280
- Walsh KA. 1970 Trypsinogens and trypsins of various species. In: *Methods of Enzymology*, vol XIX Proteolytic Enzymes. Perlmann GE, Lorand L (eds) pp 41-63 Academic Press New York and London
- Walsh KA, Wilcox PE. 1970 Serine proteases. In: *Methods in Enzymology*, vol XIX Proteolytic Enzymes. Perlmann GE, Lorand L (eds) pp 31-40 Academic Press New York and London
- Weeke B, Jarnum S. 1971 Serum concentration of 19 serum proteins in Crohn's disease and ulcerative colitis. *Gut* 12:297-302
- White R, Janoff A, Godfrey HP. 1980 Secretion of α_2 -macroglobulin by human alveolar macrophages. *Lung* 158:9-14
- Wilcox PE. 1970 Chymotrypsinogens-chymotrypsins. In: *Methods in Enzymology*, vol XIX Proteolytic Enzymes. Perlmann GE, Lorand L (eds) pp 64-108 Academic Press New York and London
- Williams WJ. 1983 Control of coagulation reactions. Williams WJ, Beutler E, Erslev AJ, Lichtman MA (eds). *Hematology* p 1247-1256

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Determination of Immunoreactive Trypsin, Pancreatic Elastase and Chymotrypsin in Extracts of Human Feces and Ileostomy Drainage

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Key Words. Antibiotic adverse effect · Trypsin · Chymotrypsin · Pancreatic elastase · Feces · Ileostomy fluid

Abstract. The total daily amount of extractable cationic trypsin, chymotrypsin, and pancreatic elastase 2 in feces and ileostomy fluids has been studied in normal individuals and healthy colectomized subjects. Quantitation was performed using immunological assays with polyethylene glycol as a fecal marker. The extractable amount of each of these enzymes in the feces of normal individuals was less than 1 mg/24 h. However, in fecal extracts from antibiotic-treated normal individuals a 100-fold increase in immunoreactive cationic trypsin was observed, while chymotrypsin and elastase 2 were only 2- to 3-fold higher. In extracts from ileostomy fluids cationic trypsin, elastase, and chymotrypsin all showed mean values in the order of 50–200 mg/24 h. The characterization of the immunoreactivity of pancreatic proteases showed no qualitative differences when measured in duodenal juice or fecal and ileostomy extracts.

The pancreatic endoproteases trypsin, chymotrypsin and elastase are secreted into the duodenum in gram quantities. The turnover of these endoproteases in the intestine is poorly understood. The bacterial flora of the large intestine is thought to play an important role in the inactivation of these proteases [1, 2].

Immunologic methods for the determination of the pancreatic endoproteases are much more sensitive and specific than enzymatic methods. We have developed specific

radioimmunological methods for the determination of immunoreactive human cationic trypsin, chymotrypsin, and pancreatic elastase 2 in serum and other body fluids [3–5]. In order to study the turnover of the pancreatic endoproteases in the intestine in health and disease in man, we have found it necessary to define normal levels of these endoproteases in the feces. This report describes the daily total amount of extractable immunoreactive cationic trypsin, chymo-

trypsin, and pancreatic elastase 2 in the feces of 9 normal subjects and in the ileostomy contents of 5 healthy colectomized subjects over a period of 3 consecutive days. Nonabsorbable polyethylene glycol (PEG-4000) was used as a fecal marker for the normal individuals [6]. Furthermore, the different immunoreactivities were characterized using gel filtration and dilution experiments.

Clinical Material

Normal Subjects. 9 healthy volunteers, aged 25–50 years, took part in the study. 5 of them had been treated with peroral antibiotics 4–12 months before the investigation. 3 individuals (S.G., K.O., C.M.) were treated with Vibramycin® (doxycycline), 100 mg daily during 7 days, because of upper respiratory tract infection. 1 subject (I.H.) was treated with Negram® (naldixic acid), 1 g four times per day for 7 days, and one (Å.L.) with Bactrim® (trimethoprim, 80 mg, and sulfamethoxazole, 400 mg) four times per day for 10 days because of urinary tract infection.

Ileostomies. 5 subjects, aged 30–60 years, having an ileostomy were studied. They all had a proctocolectomy performed 1–12 years before the investigation because of ulcerative colitis and were all in currently good health. All individuals who took part in the study gave informed consent.

Materials and Methods

PEG-4000, 0.6 g dissolved in 10 ml water, was given to the normal subjects three times daily for 10 days. Fecal samples were collected from days 8 to 10 and were of normal consistency and color in all individuals. The samples were stored at +4 °C for up to 48 h. Three spot samples were taken from different sites of each stool. Two of the samples were then weighed and 1 g was homogenized and extracted in 5 ml of ice-cold saline for 2 h. The mixture was centrifugated at 39,000 *g* for 30 min at +4 °C. After this, diisopropyl fluorophosphate (DFP), a serine protease inhibitor, was added to the supernatant at a final concentration of 10 µmol/l. This was followed by an over-

night incubation at +4 °C. Samples were then stored at –20 °C until analyzed. Immunoreactive cationic trypsin and immunoreactive pancreatic elastase 2 were determined as described earlier [3, 5]. Immunoreactive chymotrypsin was determined principally as described by Geokas et al. [4]. One sample taken from each stool was kept at –20 °C for up to 3 months and was used for PEG determination using a turbidimetric method [6]. Gel filtrations were carried out on a Sephadex G-100 column (0.9 × 35 cm) as described earlier [3].

The daily ileostomy contents from each individual were weighed and following homogenization a spot sample was extracted and handled in the same way as the fecal samples.

The daily fecal mass was determined using PEG as a marker. The total daily extractable amount of pancreatic proteases could thus be calculated from the two spot samples.

Duodenal juice was collected from healthy individuals after duodenal intubation and secretin stimulation. The juice was inactivated with DFP in the same way as the centrifugated fecal or ileostomy fluids described above.

Agarose, batch 61639, was obtained from Miles Seravac, Maidenhead, England, DFP and PEG-4000 from Merck, Darmstadt, FRG and Sephadex G-100 from AB Pharmacia Fine Chemicals, Uppsala, Sweden.

Statistics. Student's *t* test for paired observations was used.

Results

No significant differences were noted between the two samples from different sites of each stool for any of the three enzymes ($p < 0.01$; tables I, II). The extractable daily amount of pancreatic proteases in the ileostomy contents is shown in table III.

Immunoreactive Cationic Trypsin. The 24-hour amounts of extractable cationic trypsin in feces from normal subjects not treated with antibiotics were approximately 0.1–1.0 mg. In contrast, the value in antibiotic-treated subjects was about 100 times

higher (1–257 mg). The highest levels of trypsin in feces were noted in individuals treated with doxycycline. These levels were about the same as those extracted from ileostomy drainage (15–540 mg).

Immunoreactive Chymotrypsin and Elastase. There was no pronounced variation in the amount of extractable immunoreactive chymotrypsin or elastase in feces from anti-

biotic-treated subjects and subjects not treated with antibiotics. In ileostomy contents there was about 100 times more extractable immunoreactive chymotrypsin and elastase than in feces (table III).

Characterization of the Measured Immunoreactivities. The same recovery was obtained for all three enzymes (parallel dilution curves) after dilution of the DFP-inactivated

Table I. Extractable amounts of pancreatic proteases from the feces of healthy subjects

Daily fecal content g	Sample	Trypsin mg/24 h	Chymotrypsin mg/24 h	Elastase mg/24 h
180	M.B. 1A	0.2	0.18	0.04
	1B	0.4	0.18	0.05
270	2A	0.4	0.41	0.10
	2B	0.3	0.27	0.06
244	3A	0.9	0.24	0.10
	3B	1.0	0.49	0.14
242	H.W. 1A	0.6	0.47	0.5
	1B	0.6	0.68	
130	2A	0.1	0.13	0.5
	2B	0.1	0.10	
207	3A	0.1	0.08	0.5
	3B	0.1	0.08	
204	A.B. 1A	0.5	0.61	0.30
	1B	0.6	0.71	0.35
206	2A	0.8	0.86	0.32
	2B	0.9	0.72	0.38
204	3A	0.9	0.61	0.17
	3B	0.8	0.61	0.19
175	B.M.J. 1A	0.4	0.18	0.05
	1B	0.4	0.18	0.04
178	2A	0.2	0.27	0.04
	2B	0.5	0.27	0.05
239	3A	0.4	0.24	0.07
	3B	0.6	0.36	0.04
Mean $\pm$ 1 SD				
206 $\pm$ 38		0.49 $\pm$ 0.29	0.37 $\pm$ 0.24	0.19 $\pm$ 0.07

A and B represent different spot samples from the same stool sample.

Table II. Extractable amounts of pancreatic proteases in healthy subjects treated with antibiotics during the last year

Daily fecal content g	Sample		Trypsin mg/24 h	Chymotrypsin mg/24 h	Elastase mg/24 h
125	S.G.	1A	43.8	0.25	0.08
		1B	48.0	0.31	0.10
150		2A	76.5	0.38	0.09
		2B	59.0	0.30	0.06
375		3A	255.0	3.00	0.33
		3B	257.0	2.80	0.35
360	K.O.	1A	171.0	2.70	0.43
		1B	194.0	5.94	0.83
430		2A	116.0	2.58	0.22
		2B	125.0	2.37	0.21
480		3A	118.0	2.64	0.23
		3B	96.0	1.92	0.13
201	C.M.	1A	62.0	0.40	0.21
		1B	67.0	0.40	0.20
218		2A	63.0	0.55	0.30
		2B	56.0	0.55	0.27
188		3A	56.0	0.55	0.27
		3B	68.0	0.66	0.29
228	I.H.	1A	1.2	0.23	0.11
		1B	1.8	0.23	0.22
263		2A	19.0	0.79	1.08
		2B	5.0	0.56	0.53
173		3A	4.8	0.52	2.00
		3B	1.8	0.43	1.04
158	Å.L.	1A	25.2	0.32	0.07
		1B	25.8	0.40	0.14
146		2A	18.7	0.22	0.08
		2B	6.4	0.07	0.05
123		3A	9.8	0.25	0.05
		3B	11.6	0.25	0.04
Mean $\pm$ 1 SD					
241 $\pm$ 115			68.7 $\pm$ 71.3	1.09 $\pm$ 1.3	0.33 $\pm$ 0.4

A and B represent different spot samples from the same stool sample.

Table III. Pancreatic proteases in the ileostomy output of healthy colectomized subjects

Daily ileal contents g	Sample		Trypsin mg/24 h	Chymotrypsin mg/24 h	Elastase mg/24 h
1,230	K.M.	1	540	492	69
950		2	330	91	40
1,100		3	450	242	52
420	O.N.	1	133	68	136
530		2	165	66	125
500		3	64	60	100
510	S.N.	1	184	58	43
630		2	35	49	35
650		3	39	22	39
480	G.I.	1	86	24	29
680		2	226	41	35
940		3	211	15	23
1,000	G.J.	1	24	6	52
1,290		2	15	10	46
1,280		3	21	15	72
Mean $\pm$ 1 SD					
810 $\pm$ 310			168 $\pm$ 162	83.9 $\pm$ 126.7	59.7 $\pm$ 34.7

fecal extracts, duodenal juice samples and ileostomy fluid samples as after the serial dilution of the purified DFP-inactivated pancreatic proteases used in the radioimmunoassays. On gel filtration of fecal extracts, ileostomy contents and duodenal juice samples, all trypsin-, elastase- and chymotrypsin-like immunoreactive material eluted in a single peak corresponding to the molecular weight of the native enzymes.

Discussion

In earlier investigations it has been demonstrated that analyses of pancreatic enzymes in spot stool samples corresponded

closely to the values of these enzymes in aliquots of homogenized 24-hour stool collections [7, 8]. In the present study, using PEG-4000 as a marker, there is a close correlation between the two spot samples taken from each stool. This demonstrates that spot-sampling is reliable in studies of this design.

The dilution experiments suggest a close antigenic resemblance between the inactivated purified pancreatic proteases used as standards and the measured immunoreactive inactivated enzymes in duodenal juice and fecal extracts. This is further documented by the gel filtration studies, showing that the different immunoreactive enzymes had the same molecular size in fecal extracts as in duodenal juice. Thus, enzymes maintaining

their immunogenic sites do not change qualitatively during their passage through the intestine. However, in this study the amount of active enzymes was not determined, as only immunochemical methods were used.

The daily production of pancreatic cationic trypsin and chymotrypsin can be roughly estimated as 1–3 g of each [9]. For pancreatic elastase 2 the daily production is about 0.5 g [9]. The daily levels of these proteases in fecal extracts are in the order of 0.1–1 mg for each of these enzymes in normal individuals not treated with antibiotics. This means that normally less than 0.1% of these enzymes can be extracted from feces after passage through the intestinal tract. Earlier experimental studies have shown that fecal extracts from man [11] and rat [1] contain only minimal amounts of pancreatic proteases measured by both enzymatic and immunologic methods. The extractable amounts of pancreatic trypsin, chymotrypsin and elastase 2 in ileostomy contents are in the order of 20–500 mg/day, which is about 10–30% of the total daily pancreatic production. These results suggest that a large amount of the pancreatic endopeptidases either is degraded in the small intestine or is bound to particles in the centrifugate. A substantial amount of these enzymes is, however, also degraded in the colon normally. In earlier experimental studies, it has been demonstrated that fecal extracts from germfree rats contained large amounts of pancreatic trypsin and elastase. High levels of these enzymes have also been demonstrated in feces from man and normal rats after peroral antibiotic treatment [1, 2, 10, 11]. In the present study it has been demonstrated that fecal extracts from doxycycline-treated individuals contain about the same amount of trypsin as extracts of ileostomy contents. These re-

sults show the importance of the colonic bacterial flora in the degrading of pancreatic endopeptidases. An alteration of the bacterial flora caused by antibiotic treatment probably makes it possible for an increased amount of the trypsin reaching the colon to pass through it without further degradation. Diminished trypsin degradation as a result of decreased transit time is a less likely explanation because of the low amounts of elastase and chymotrypsin present.

Experimental studies in the rat demonstrated large amounts of pancreatic proteases in feces as early as 2 days after initiating peroral antibiotic treatment [10]. Thus, the onset of the effect of antibiotics on the intestinal flora seems to be very rapid. It was also demonstrated that this antibiotic-induced effect on the colonic bacterial flora is long-lasting even after a short period of peroral treatment [10]. Normal degradation of pancreatic proteases was not observed until an enema of fecal suspension from nontreated animals was given [10]. The long-lasting effect of peroral antibiotic treatment on the colonic flora in this respect is further demonstrated in the present study, as none of the antibiotic-treated subjects had taken any of these drugs for at least 4 months prior to fecal sampling.

Different mechanisms seem to exist for the degradation of the different proteases, as indicated by the long-lasting effect of antibiotics on trypsin degradation as compared to the short-term effects on both trypsin and elastase degradation found in an earlier study [11].

The level of pancreatic chymotrypsin and trypsin in feces studied by enzymatic methods has been used in many laboratories as a pancreatic function test [12, 13]. The determination is made on fecal homogenates using

titrimetric methods. In these investigations much higher values for fecal chymotrypsin and trypsin are found than in the present study, where analyses were made in supernatants. The explanation is probably that large amounts of the pancreatic endopeptidases are adsorbed to intestinal cells or particles and pass the small intestine without loss of activity. This adsorption seems to be very strong and does not change during large intestinal transit [14, 15]. The apparent high levels of pancreatic proteases measured on fecal homogenates with titrimetric methods should, however, be judged with some caution. Enzymatic techniques lack complete specificity and particle-bound enzymes may show altered kinetics versus substrates as compared to the free enzyme. Furthermore, bacterial enzymes might contribute to the activity measured [16–18]. Specific immunologic techniques circumvent several of these problems. Furthermore, they apparently give reproducible results for the dominating pancreatic proteases, when applied on standardized extracts of intestinal content or feces.

This study has shown that normally very small amounts of pancreatic endopeptidases are extractable from feces. As in earlier experimental studies, we have clearly demonstrated that antibiotic treatment causes an impairment of the normal bacterial degradation of pancreatic proteases in the colon. It has also been shown that PEG-4000 is a useful marker allowing fecal spot samples to be used for analyses instead of 24-hour collections.

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References

- 1 Genell, S.; Gustafsson, B.; Ohlsson, K.: Quantitation of active pancreatic endopeptidases in the intestinal contents of germfree and conventional rats. *Scand. J. Gastroent.* 11: 757–762 (1976).
- 2 Genell, S.; Gustafsson, B.; Ohlsson, K.: Immunochemical quantitation of pancreatic endopeptidases in the intestinal contents of germfree and conventional rats. *Scand. J. Gastroent.* 12: 811–820 (1977).
- 3 Borgström, A.; Ohlsson, K.: Radioimmunological determination and characterization of cathodal trypsin-like immunoreactivity in normal human plasma. *Scand. J. clin. Lab. Invest.* 36: 809–814 (1976).
- 4 Geokas, M.; Largman, C.; Brodrick, J.; Johnson, J.; Fassett, M.: Immunoreactive forms of human pancreatic chymotrypsin in normal plasma. *J. biol. Chem.* 254: 2775–2781 (1979).
- 5 Borgström, A.; Kukora, J.; Ohlsson, K.: Studies on immunoreactive pancreatic elastase 2 in human serum. *Hoppe-Seyler's Z. physiol. Chem.* 361: 633–640 (1980).
- 6 Malawer, S.; Powell, D.: An improved turbidimetric analysis of polyethylene glycol utilizing an emulsifier. *Gastroenterology* 53: 250–256 (1967).
- 7 Ammann, R.; Kashiwagi, H.: Pancreatic exocrine insufficiency and proteolytic enzymes in stool. *Helv. med. Acta* 33: 220–228 (1966).
- 8 Haverback, B.J.; Dyce, B.J.; Gutentag, J.; Montgomery, D.W.: Measurement of trypsin and chymotrypsin in stool. *Gastroenterology* 44: 588–597 (1963).
- 9 Kukral, J.; Adams, A.; Preston, F.: Protein producing capacity of the human exocrine pancreas. *Surgery* 162: 67–73 (1965).
- 10 Genell, S.; Gustafsson, B.: Impaired enteric degradation of pancreatic endopeptidases in antibiotic-treated rats. *Scand. J. Gastroent.* 12: 801–809 (1977).

- 11 Borgström, A.; Genell, S.; Ohlsson, K.: Elevated fecal levels of endogenous pancreatic endopeptidases after antibiotic treatment. *Scand. J. Gastroent.* 12: 525-529 (1977).
- 12 Sale, K.; Goldberg, B.; Thjodleifson, B.; Wormsley, K.: Trypsin and chymotrypsin in duodenal aspirate and feces in response to secretin and cholecystokinin-pancreozymin. *Gut* 15: 132-138 (1974).
- 13 Ammann, R.; Togwercher, E.; Kaishiwagi, H.; Rosenmund, H.: Diagnostic value of fecal chymotrypsin and trypsin assessment for determination of pancreatic disease. *Am. J. dig. Dis.* 13: 123-146 (1968).
- 14 Dürr, H.; Schneider, R.; Bode, C.; Bode, J.: Fecal chymotrypsin: study on some characteristics of the enzyme. *Digestion* 17: 396-403 (1978).
- 15 Goldberg, D.M.; Campell, R.; Roy, A.D.: Studies on the binding of trypsin and chymotrypsin by human intestinal mucosa. *Scand. J. Gastroent.* 4: 217-226 (1969).
- 16 Pacaud, M.: Protease II from *Escherichia coli*. *J. biol. Chem.* 250: 7771-7779 (1975).
- 17 Pacaud, M.; Sibilli, L.; Le Bras, G.: Protease I from *Escherichia coli*. *Eur. J. Biochem.* 69: 141-151 (1976).
- 18 Pacaud, M.: Purification of protease II from *Escherichia coli* by affinity chromatography and separation of two enzyme species from cells harvested at late log phase. *Eur. J. Biochem.* 64: 199-204 (1976).

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METABOLISM OF ^{131}I -LABELLED HUMAN PANCREATIC CATIONIC
TRYPSIN AFTER INTRADUODENAL ADMINISTRATION

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Abstract

Human pancreatic cationic trypsin labelled with ^{131}I was administered into the duodenum in nine healthy individuals. Five had earlier been proctocolectomized and had ileostomies. Radioactivity was measured in plasma, urine, ileostomy content and faeces for a period of 72 hours. Radioactivity was present in plasma 15 minutes after administration. The total recovery of radioactivity was 78-98%, the largest amount being observed in urine during the first 24-hour period. About 20% of the administered radioactive dose was recovered in ileostomy content and 10% in faeces. The recovered radioactivity in plasma, urine and extracts of ileostomy content and faeces was characterized with dialysis and gel-filtration. All radioactivity in plasma and urine corresponded to free ^{131}I , whereas in the extracts radioactivity corresponding to intact enzyme and degradation products as well as a small amount of free ^{131}I was observed. It is concluded that pancreatic trypsin is degraded during intestinal passage as with other proteins. Due to a de-iodinating mechanism in the intestine, only free ^{131}I is absorbed into the circulation. This de-iodination does not take place in duodenal juice. The absence of high-molecular-weight radioactivity in plasma argues against an enteropancreatic circulation.

Key words: Faeces, ileostomy content, pancreatic peptidases, trypsin, de-iodase

Introduction

The fate of activated pancreatic endopeptidases in the intestinal canal has been investigated often but with rather conflicting results (1,2,3,4). This might be due to the use of different analytic techniques or different species. It has been demonstrated that most of the pancreatic enzymes are degraded in the small intestine (5,6,7). Alternative metabolic pathways for the pancreatic endopeptidases have been discussed, such as enteropancreatic circulation (8,9,10), inactivation by the intestinal microflora (6,11) or binding to inte-

stinal particles (12,13).

Radio-labelled substances have often been used in studies on the metabolism of pancreatic substances. However, little consideration has been given to the influence of possible de-iodases in the intestinal wall or intestinal content (14), a pertinent question as radio-iodinated substances have been used in most studies.

The aim of the present study was to follow the fate of ^{131}I -labelled human cationic trypsin after introduction into the duodenum. Special attention was paid to the possible resorption of undegraded labelled trypsin, the binding to particular substances in the intestine and the role of the colon in the degradation of non-particle-bound labelled trypsin. The possible influence of de-iodases was elucidated by analysis for tyrosine-bound and free ^{131}I .

Subjects and Materials

Subjects. Nine persons, 35-50 years old, took part in the study. Four of them were healthy members of the staff. Five persons had undergone proctocolectomy as a result of ulcerative colitis at least two years earlier, but were now in perfect health with a well-functioning ileostomy. One of the non-operated persons had taken Bactrim^R (Trimetoprim 80 mg and Sulfametoxazol 400 mg) four times per day for 10 days, because of a urinary tract infection one month prior to the investigation. All individuals were treated with Solvejod^R (potassium iodide 0.9 g/day) during 4 days, starting the day before the infusion of the labelled substance. Informed consent was obtained from all participants in the study and the project was approved by the Ethical Committee for Research Projects, Faculty of Medicine, University of Lund.

Specific materials: Na ^{131}I and Na ^{125}I were obtained from the Radiochemical Centre, Amersham, England. Sephadex G-10, G-25, G-75 and G-100 were obtained from AB Pharmacia Fine Chemicals, Uppsala, Sweden. Di-iso-propyl-fluorophosphate (DFP)

was obtained from Merck, Darmstadt and N-alpha-benzoyl-DL-arginine-4-nitroanilide HCl (BAPNA) was purchased from Fluka AG, Switzerland. 3-iodo-L-tyrosine was obtained from the Sigma Chemical Company, USA. Dialyzing tubes with a cut-off of about MW 10,000 daltons were obtained from Union Carbide, Chicago, Illinois, USA. Duodenal juice was obtained from a healthy individual.

Methods

Human pancreatic cationic trypsin was purified as previously described (15) and labelled with ^{131}I using a lactoperoxidase method (16). The labelled enzyme was applied to a Sephadex G-25 column (1.8 x 5 cm) equilibrated and eluted with 0.05M sodium acetate buffer, 0.5M NaCl, pH 4.3 to separate the labelled enzyme from free iodine. The protein-containing fractions were pooled and applied to a G-75 column (1.6 x 20cm) eluted with the same buffer to separate labelled trypsin from lactoperoxidase. The protein fractions with the highest activity were pooled. The specific activity was 5-10 $\mu\text{Ci}/\mu\text{g}$ protein. The biological activity of the labelled trypsin was tested by incubating 1 μg of labelled trypsin and 1 μg of DFP inactivated labelled trypsin, each with 500 μl serum for 15 minutes followed by gel-filtration on a G-100 column (0.9 x 60 cm) equilibrated with 0.05M Tris-HCl buffer, 0.5M NaCl, pH 7.4. The radioactivity was then measured in the fractions and characterized utilizing antibodies against $\alpha_2\text{-M}$ and $\alpha_1\text{-AT}$ as earlier described (17). The enzymatic activity of ^{131}I -trypsin was also tested with BAPNA as a substrate (18).

All subjects were required to fast for at least 8 hours prior to the investigation. A duodenal tube was positioned with the tip approximately at the level of the papilla of Vater under fluoroscopic control. Twenty-five μCi of labelled enzyme in 5 ml of buffer solution was instilled. The tube was flushed with 100 ml saline after the infusion.

From a peripheral vein, blood samples were drawn into test tubes containing EDTA 30 minutes before and 15, 30, 60, 120 minu-

tes and 24, 48 and 72 hours after the instillation of the labelled enzyme into the duodenum. After centrifugation plasma was separated and used for further analysis. All urine, faeces and ileostomy contents were collected for a period of 3 days after the administration of the enzyme. The samples from each 24-hour period were kept separate. Faeces from the 4 non-operated persons were normal in consistency and colour. After homogenization of faeces or ileostomy contents from the different 24-hour periods 2 g spot samples were homogenized in 10 ml of saline at 4°C. After extraction for 2 hours at 4°C, the samples were centrifuged at $39,000 \times g$ for 30 minutes at +4°C. Supernatants and pellets were carefully separated. Two ml of each plasma sample, urine sample and each supernatant from faecal or ileostomy contents were dialyzed against 2x2.5 l of saline for 24 hours. The radioactivity in the dialyzed and non-dialyzed samples was measured.

Characterization of the low-molecular-weight radioactive substances was made with gel-filtration on G-10 and G-25 columns (0.9 x 20 cm), equilibrated with 0.05M Tris-HCl buffer, 0.15M NaCl, pH 7.0, after adding 0.3 pmol Na^{125}I (71 MBq/nmol) to the different samples. One ml of the following samples was analysed: plasma, urine, and supernatants from faeces. Ten ml duodenal juice were incubated with 0.1 μg ^{131}I -trypsin for 1 hour and 24 hours at 20°C followed by gel-filtration on a G-10 column, 0.9x20 cm, equilibrated and eluted with the same buffer as above.

A mixture of 0.3 pmol Na^{125}I and 1 mg mono-I-tyrosine was incubated in 0.05M Tris-HCl, 0.5M NaCl, pH 7.0 and was then gel-filtrated on G-10 and G-25 columns, eluted with the same buffer. The mono-I-tyrosine was assayed as absorbance at 280 nm.

Radioactivity measurements. Total daily urine, faecal or ileostomy drainage radioactivity was measured in a lead-shielded scintillation detector (4"x3" with Camberra electronics). So that measurements were always carried out on full containers, the urine and ileostomy drainage containers from

each 24-hour period were filled up with water, to a total volume of 2 l before measurement. The containers with faeces were filled with soft paper before measurement. The measurement was done both at the top and the bottom of the containers and the mean value of the two measurements was used for calculations. Radioactive levels in plasma samples, ileostomy and faecal supernatants and pellets were measured in a well-type scintillation detector with two channels (LKB Wallac 1280).

Results

Specificity and biological activity of the labelled enzyme

According to the BAPNA-splitting activity 90% of the labelled trypsin was active. After gel-filtration of the labelled trypsin incubated with serum the radioactivity was eluted to 80% in the void volume corresponding to α_2 -macroglobulin-trypsin complexes and 20% in a volume corresponding to α_1 -anti-trypsin complexes. The radioactivity was precipitated by antibodies against α_2 -M and α_1 -AT. No low-molecular-weight radioactivity corresponding to inactive or degraded trypsin was seen. After gel-filtration of DFP-inactivated ^{131}I -trypsin, incubated with serum, all the radioactivity eluted in a volume corresponding to the molecular size of trypsin.

Recovery of administered radioactivity

The total recovery of radioactivity during 72-hours was 78-98% (mean 90%) in the group of normal individuals and 84-97% (mean 92%) in the group of colectomized subjects. In all individuals, the largest amount of recovered radioactivity was observed in urine (48-91%) during the first 24-hour period (42-74%) (Fig.1).

Radioactivity measurements of plasma

In all individuals, labelled substance was observed in plasma after 15 minutes (Fig. 2). The highest value of radioactivity in plasma was observed 1 hour after the duodenal infusion of ^{131}I -trypsin and remained on this level for 2 hours. After 72 hours no radioactivity was observed in plasma. There was no significant difference between the plasma radioactivity

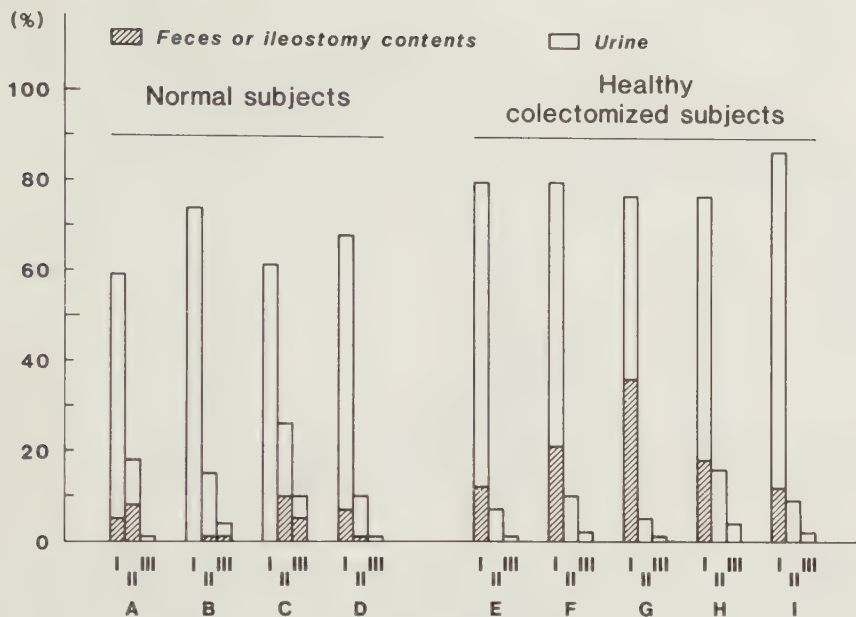


Fig. 1 Daily recovery of ^{131}I in faeces, ileostomy drainage and urine calculated as per cent of the administered dose. The capital letters refer to the different subjects. Roman numerals represent the three consecutive 24-hour periods after the administration of ^{131}I -trypsin.

measured in the normal individuals and in subjects who had undergone colectomy. After dialysis of plasma samples no radioactive substance could be demonstrated in any plasma sample in the normal or in the colectomized group.

Radioactivity measurements on ileostomy contents

Almost all of the recovered radioactivity in the ileostomy contents was observed during the first 24-hour period and was 13-35% (mean 19%) of the administered radioactive dose. Only trace amounts of the radioactive substance were observed in the second 24-hour period and none during the third period (Fig. 1). The distribution of radioactivity (after extraction and centrifugation), expressed as per cent of the instilled dose, is shown in Fig. 3. 4.7-14% (mean 8%) of the administered dose was found in the pellets and 6-25% (mean 11%) in the supernatant. 1-7% (mean 4%) of the administered dose was not dialyzable after extraction.

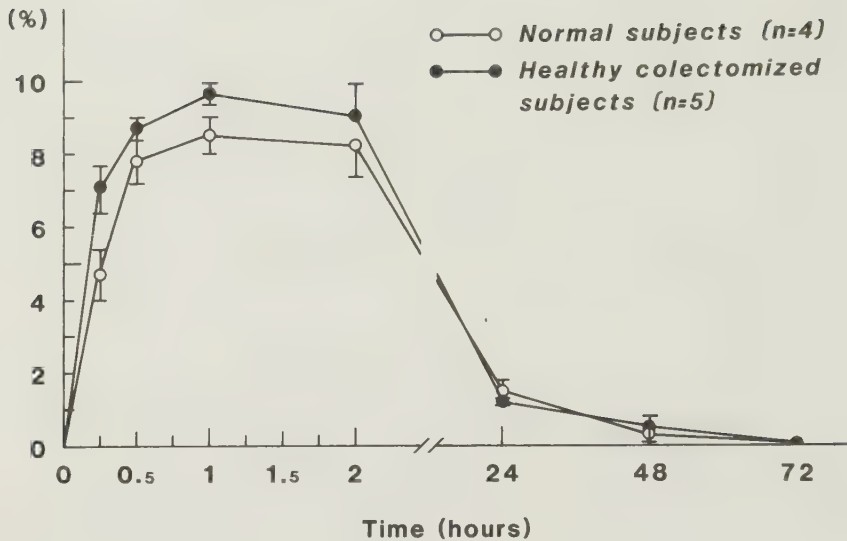


Fig. 2 The percentage of ^{131}I of administered intraduodenal dose of ^{131}I -trypsin in plasma (mean $\pm$ SEM). Total plasma volume calculated as 42 ml/kg body weight.

Radioactivity measurements in faeces

The recovery of instilled radioactivity in faeces from the four normal individuals during the 72-hour period was 2.5-14% (mean 9%). Except in one person, most of the faecal recovery was observed during the second 24-hour period (Fig. 1). After extraction and centrifugation of faecal samples, 2-12% (mean 7%) of the instilled radioactivity was found in the pellets and 0.6-4.3% (mean 3%) in the supernatant (Fig. 3). In three of the subjects, 0.1-0.6% (mean 0.5%) of the given dose was present as nondialyzable high-molecular-weight-bound radioactivity in the faecal supernatants. However, in the fourth individual (A), treated with trimetoprim-sulfa prior to the study, the nondialyzable high-molecular-weight-bound radioactivity in faecal supernatants was 3.5% of the instilled dose.

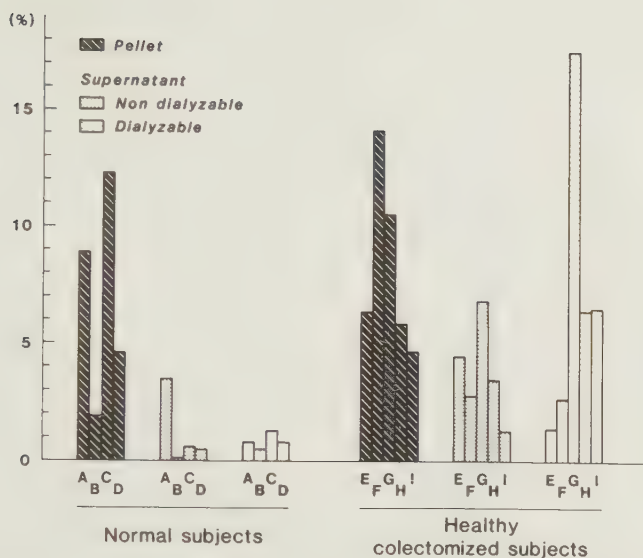


Fig. 3 Distribution of radioactivity in faeces and ileostomy content after centrifugation divided in high-molecular-weight radioactivity (non dialyzable), low-molecular-weight-bound radioactivity (dialyzable) and particle-bound radioactivity (pellet), expressed as percent of the administered intraduodenal ^{131}I -trypsin. Capital letters refer to different subjects. Subject A was treated with peroral antibiotics prior to the investigation.

Characterization of the radioactivity

The recovered radioactive substances in plasma and urine samples were eluted in the same volume as Na^{125}I according to the gel-filtration (Fig. 4) and in a smaller volume than mono-I-tyrosine. The radioactivity in extracts from faecal samples eluted in three peaks; one in the void volume corresponding to high-molecular-weight substances, one in the same volume as Na^{125}I , and one in a volume corresponding to mono-I-tyrosine (Fig. 5). The recovered labelled substances from duodenal juice, incubated with the labelled trypsin for 1 hour, eluted in two peaks, one corresponding to high-molecular-weight substances and one corresponding to mono-I-tyrosine. After incubation of duodenal juice and labelled trypsin for 12 hours the high-molecular-weight-bound radioactivity peak decreased and the low-molecular-weight peak, corresponding mainly to mono-I-tyrosine, increased (Fig. 6).

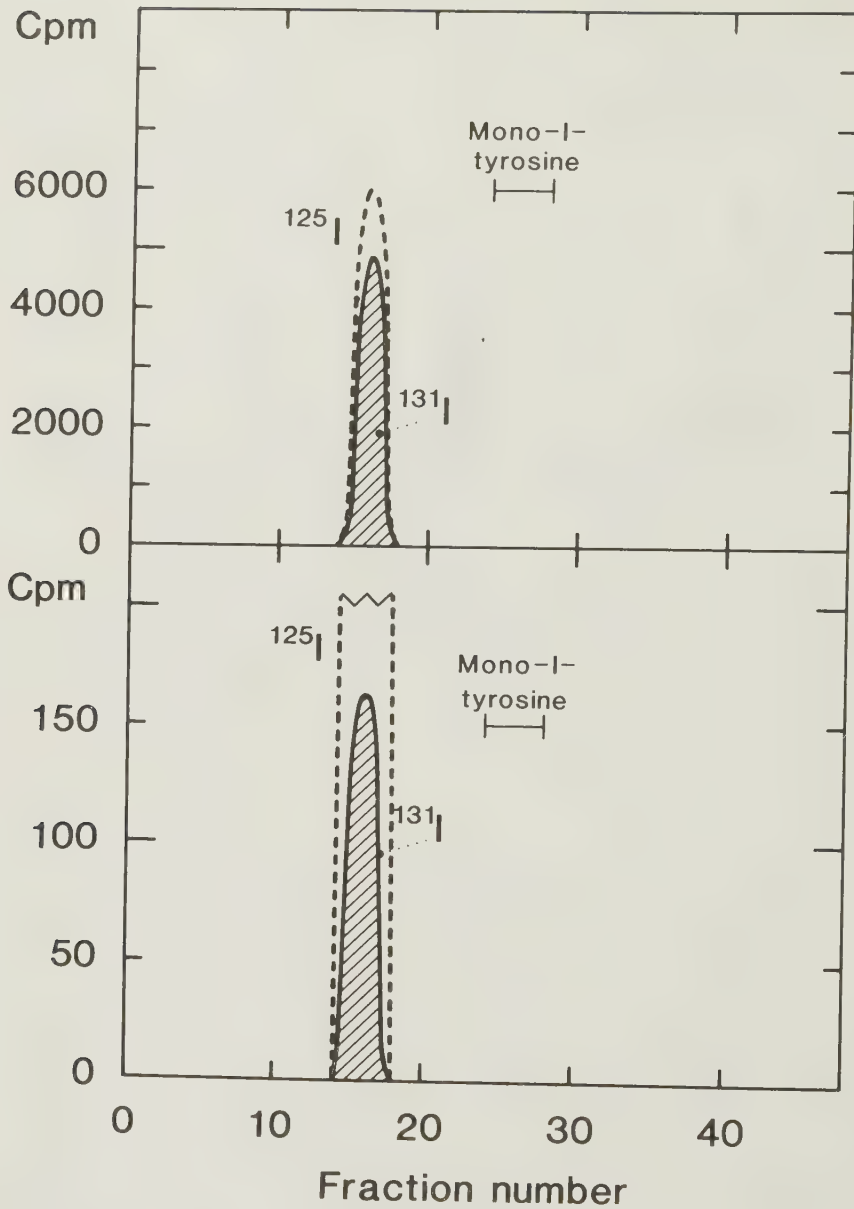


Fig. 4 Chromatographic characterization of radioactivity ^{131}I (—) in urine (upper part) from the first day and plasma samples (lower part) 60 min after the intraduodenal instillation of the labelled trypsin. (Sephadex G-10, 0.9x20 cm). The elution volumes of ^{125}I (---) and mono-I-tyrosine are given for reference. Radioactivity was measured as CPM/ml. All the radioactivity was eluted as free iodine. (For details see Methods).

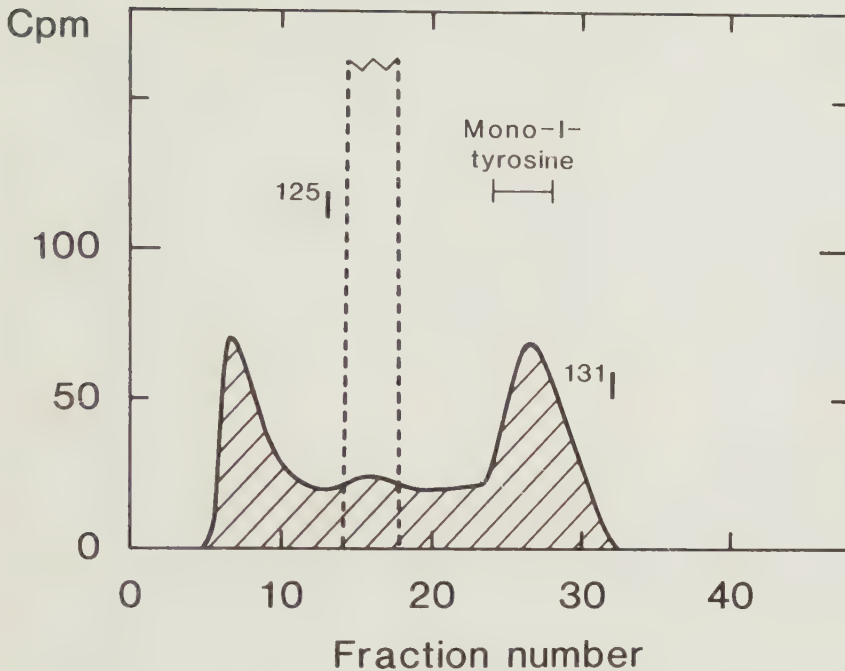


Fig. 5 Chromatographic characterization of extractable radioactivity ^{131}I (—) in faecal samples the day after instillation of ^{131}I -labelled trypsin (Sephadex G25, 0.9x20 cm). The elution volumes of ^{125}I (---) and mono-I-tyrosine are given for reference. Radioactivity was measured as CPM/ml. The ^{131}I radioactivity eluted in the void volume and in a volume corresponding to mono-I-tyrosine. (For details see Methods).

Discussion

In the present study ^{131}I -labelled biologically active cationic trypsin was introduced into the duodenum in order to study its metabolism in the intestinal tract. The biological activity of the labelled enzyme was ascertained by showing its ability to bind to the major protease inhibitors and to cleave BAPNA.

During the passage through the small intestine 80% of the administered radioactivity must have been absorbed as only 20% was recovered in the ileostomy drainage. Labelled substances

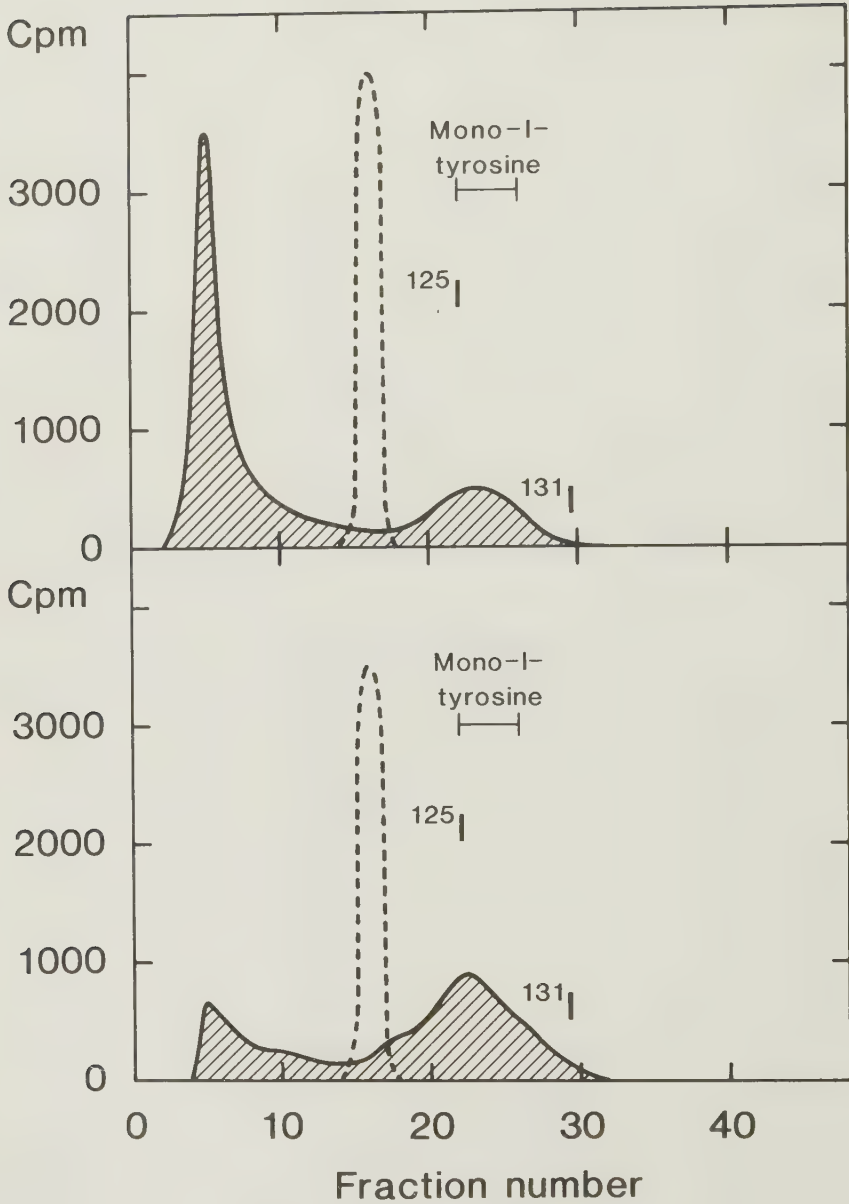


Fig. 6 Chromatographic characterization of radioactivity ^{131}I (—) in duodenal juice after incubation of 10 ml of duodenal juice with 0.1 mg ^{131}I -trypsin for 1 hour (upper part), and 12 hours (lower part) (Sephadex G-10, 0.9x20 cm). The elution volumes of ^{125}I (---) and mono-I-tyrosine are given for reference. Radioactivity was measured as CPM/ml.

appeared in the circulation as soon as 15 minutes after the intraduodenal instillation of the ^{131}I -trypsin. A maximum was reached after 1 hour with a 1-hour duration. All the radioactivity in plasma was dialyzable and, according to the results of gel-filtration experiments, of the same molecular size as NaI . The amount of nonextractable radioactivity in faeces and ileostomy content was about 8% of the administered dose in both materials. This radioactivity might represent enzyme molecules absorbed by desquamated small intestinal cells, thereby passing the large intestine without further degradation (12).

The extractable radioactivity in ileostomy content and faeces separated into three peaks on gel-filtration. Dialysis experiments demonstrated that a high-molecular-weight radioactive substance, probably representing undegraded trypsin, accounted, in ileostomy extracts, for about 4%, and in faecal extracts 0.1-0.6% of the intraduodenally instilled dose. A minor peak of free ^{131}I might be the result of intraluminal deiodases or rather the secretion of ^{131}I into the gastrointestinal tract, while a major third peak was identified as mono-I-tyrosine-bound ^{131}I , a result of degradation of the instilled labelled trypsin. It was obvious that the larger part of the instilled ^{131}I -trypsin was degraded and absorbed in the small intestine as ordinary proteins, in agreement with the findings of earlier studies (5). The labelled molecules resulting from intestinal degradation are apparently deiodinated during the passage through the intestinal wall. Direct incubation of duodenal juice and ^{131}I -trypsin resulted in the production of labelled degradation products corresponding to mono-I-tyrosine but virtually no free iodine. Studies in suckling rats have shown that radio-labelled mono-I-tyrosine and di-I-tyrosine are deiodinated in the intestinal cells (14), which supports our observation. Furthermore, studies on the possible absorption of ^{131}I -labelled albumin in the rat have shown the recovered radioactivity in serum to be free iodine in accordance with our results (19). Neither pancreatic extract, extracts from rat intestinal mucosa nor gastric juice could deiodinate the labelled pro-

tein. A prerequisite for studies such as these is the effective blocking of the I-binding sites in the organism by giving non-labelled NaI before the study (20).

Using immunochemical methods we found earlier about 10% of the total daily pancreatic production of cationic trypsin in ileostomy contents and about 0.1% in faecal extracts (6). These figures roughly correspond to the recovery of nondialyzable extractable radioactive substances in the supernatants mentioned above. The difference in recovery of undegraded trypsin in ileostomy content and faecal extracts must reflect a further degradation of the enzyme in the large intestine probably caused by the colonic bacterial flora (6,11). Accordingly peroral antibiotics might disturb this mechanism (21). One person in the present study, who was recently treated with antibiotics, showed an increased faecal excretion of undegraded trypsin corresponding to the amount seen in ileostomy fluids. Thus, only a small part (10%) of the totally produced pancreatic trypsin is degraded in the large intestine by colonic bacteria and normally only trace amounts are demonstrated in faecal extracts. However, the amounts of trypsin demonstrated in the faecal supernatants might still be 50 times greater after antibiotic treatment compared with normal levels, which can be explained by an inhibition of the colonic degradation. The pathophysiological significance of this observation is still not understood.

Recently, an enteropancreatic circulation of pancreatic enzyme has been proposed in man (8,10). Intravenously or intraduodenally given radio-labelled human trypsin was claimed to be fully recovered in pancreatic juice (8,10). In the present study the intraduodenally administered amount of radio-labelled enzyme was larger than in earlier studies on the metabolism of infused human trypsin (8,10). Still we found no trace of high-molecular-weight-bound ¹³¹I corresponding either to absorbed undegraded trypsin or trypsin in complex with plasma protease inhibitors in any plasma samples from the 9 individuals. The exclusion limit for an enteropancreatic recirculation in our study is 0.5%. A daily reabsorption of only 0.1%

of all trypsin excreted to the intestine could account for the normal levels of immunoreactive trypsin found in serum (22). However, the immunoreactive trypsin in serum consists of trypsinogen and cannot originate from the intestine. The purpose of this study was to rule out a major reabsorption of trypsin that could be a substantial part of an enteropancreatic recirculation as a conservation mechanism. Earlier investigations in our laboratory with immunochemical techniques have failed to show any increase in the plasma level of trypsin or trypsin in complex with α_1 -antitrypsin after stimulation of the pancreatic gland in healthy individuals (22). Thus it seems less likely that radio-labelled trypsin in the normal intestine, as in the present study, is de-iodinated by intestinal mucosal cells and then resorbed in intact form. A limited leakage into the blood of active trypsin might occur in gastro-duodenitis through a destroyed intestinal mucosa, as indicated by our finding of high levels of trypsin in complex with α_1 -AT in this condition (unpublished data). The resorbed trypsin is immediately bound by the plasma protease inhibitors α_2 -M - the major part - and α_1 -AT. Such inhibitor-bound trypsin is, however, then rapidly eliminated - mainly in the RESsystem (23).

In conclusion, it has been demonstrated that the largest amount of pancreatic trypsin is normally degraded during the passage through the small intestine. The early appearance of radioactivity in plasma indicates that the degradation starts in the most proximal small intestine. Particle-bound enzyme passes the colon without further degradation and undegraded free trypsin molecules reaching the large intestine are normally further degraded by the colonic bacterial flora. The labelled molecules resulting from the intestinal degradation are de-iodinated during the passage through the intestinal wall. There are no signs of an enteropancreatic circulation.

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References

1. Borgström, B.; Dahlqvist, A.; Gustafsson, B.E.; Lundh, G.; Malmqvist, J.: Trypsin, invertase and amylase content in feces of germfree rats. *Proc. Soc. Exp. Biol. Med.* 102:154-155 (1959).
2. Haverback, B.J.; Dyce, B.J.; Gutentag, P.J.; Montgomery, D.W.: Measurement of trypsin and chymotrypsin in stool. *Gastroenterology* 44:588-596 (1963).
3. Pelot, D.; Grossman, M.I.: Distribution and fate of pancreatic enzymes in the small intestine of the rat. *Am. J. Physiol.* 202:285-288 (1962).
4. Goldberg, D.M.; Campell, R.; Roy, A.D.: Fate of trypsin and chymotrypsin in the human small intestine. *Gut* 10:477-483 (1969).
5. Borgström, B.; Dahlqvist, A.; Lundh, G.; Sjövall, J.: Studies on intestinal digestion and absorption in the human. *J. Clin. Invest.* 36:1521-1536 (1957).
6. Bohe, M.; Borgström, A.; Genell, S.; Ohlsson, K.: Determination of immunoreactive trypsin, pancreatic elastase and chymotrypsin in extracts of human feces and ileostomy drainage. *Digestion* 27:8-15 (1983).
7. Roy, A.D.; Campell, R.; Goldberg, D.M.: Effect of diet on the trypsin and chymotrypsin output in the stools of patients with an ileostomy. *Gastroenterology* 53:584-589 (1967).

8. Lake-Bakaar, G.; Rubio, C.F.; Mc Kavanagh, S.; Potter, B.J.; Summerfield, J.A.: Metabolism of ^{125}I -labelled trypsin in man: evidence for recirculation. *Gut* 21: 580-586 (1980).
9. Götze, H.; Rothman, S.S.: Enteropancreatic circulation of digestive enzyme as a conservative mechanism. *Nature* 257:607-609 (1975).
10. Heinrich, H.C.; Gabbe, E.E.; Brüggemann, J.; Icagic, F.; Classen, M.: Enteropancreatic circulation of trypsin in man. *Klin. Wochenschrift* 57:1295-1297 (1979).
11. Genell, S.; Gustafsson, B.E.; Ohlsson, K.: Immunochemical quantitation of pancreatic endopeptidases in the intestinal contents of germfree and conventional rats. *Scand. J. Gastroent.* 12:811-820 (1977).
12. Goldberg, D.M.; Campell, R.; Roy, A.D.: Studies on the binding of trypsin and chymotrypsin by human intestinal mucosa. *Scand. J. Gastroent.* 4:217-226 (1969).
13. Dürr, H.K.; Schneider, R.; Bode, C.; Bode, J.C.: Fecal chymotrypsin: study on some characteristics of the enzyme. *Digestion* 17:396-403 (1978).
14. Jones, R.E.: De-iodination of labelled protein during intestinal transmission in the suckling rat. *Proc. R. Soc. (Ser. B)* 199:279-290 (1977).
15. Ohlsson, K.; Skude, G.: Demonstration and semiquantitative determination of complexes between various proteases and human α_2 -macroglobulin. *Clinica Chim. Acta* 66:1-7 (1976).
16. Thorell, J.I.; Johansson, B.G.: Enzymatic iodination of polypeptides with ^{125}I to high specific activity. *Biochim. Biophys. Acta* 251:363-467 (1971).
17. Genell, S.; Ohlsson, K.; Wehlin, L.: The fate of ^{131}I -labelled human pancreatic elastase after intraduodenal administration. *Hoppe-Seyler's Z. Physiol. Chem.* 358:115-121 (1977).
18. Erlanger, B.F.; Kokowsky, N.; Cohen, W.: The preparation and properties of two new chromogenic substrates of trypsin. *Arch. Biochem. Biophys.* 95:271-278 (1961).

19. Parkins, R.A.; Dinitriadou, A.; Booth, C.C.: The rates and sites of absorption of ^{131}I -labelled albumin and sodium ^{131}I in the rat. *Clin. Sci.* 19:595-604 (1960).
20. Skogh, T.: Overestimate of ^{125}I protein uptake from adult mouse gut. *Gut* 23:1077-1080 (1980).
21. Borgström, A.; Genell, S.; Ohlsson, K.: Elevated fecal levels of endogenous pancreatic endopeptidases after antibiotic treatment. *Scand. J. Gastroent.* 12:525-529 (1977).
22. Borgström, A.; Ohlsson, K.: Studies on the turnover of endogenous cathodal trypsinogen in man. *Eur. J. Clin. Invest.* 8:379-382 (1978).
23. Ohlsson, K.: Elimination of ^{125}I -trypsin-alpha-macroglobulin complexes from blood by the reticuloendothelial cells in dog. *Acta Physiol. Scand.* 81:269-272 (1971).

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Fate of Intravenously Injected Trypsin in Dog with Special Reference to the Existence of an Enteropancreatic Circulation

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Key Words. Alpha₁-antitrypsin · Alpha-macroglobulin · Bile · Pancreatic juice · Trypsin

Abstract. In 3 mongrel dogs the pancreatic duct and bile duct were cannulated. After intravenous injection of ¹²⁵I-labelled anionic dog trypsin bile, pancreatic juice and blood samples were collected during 3 h. ¹²⁵I-trypsin is eliminated from the circulation in dogs with a half time of 15 min. Only 0.15% of the radioactivity is secreted in the pancreatic juice and 0.75% in the bile during 3 h. 10% of the radioactivity in pancreatic juice is protein bound and 45% in the bile is protein bound. The results argue against biologically significant enteropancreatic circulation of trypsin in dogs.

The pancreatic endopeptidases, i.e., trypsin, chymotrypsin and elastase, account for a major portion of the protein secreted in the pancreatic juice. The fate of these enzymes after their activation is poorly understood. Only small amounts are normally eliminated in the feces [1]. Several metabolic pathways are possible: degradation by autodigestion or bacterial proteases, binding to inhibitors or to intestinal cells or to bacteria. Recently several authors have suggested an absorption of intact pancreatic enzymes and even postulated an enteropancreatic circulation as a

'conservation mechanism' for these enzymes [2, 3].

Such an enteropancreatic circulation of pancreatic enzymes, if existing, could be separated in several steps: first, resorption or transport of active enzymes from the intestinal lumen into the blood stream, second, transport in the blood and third, uptake from blood and secretion in pancreatic juice.

The present investigation deals with the possible uptake and secretion of ¹²⁵I-labelled anionic trypsin in dogs from the blood to the pancreatic juice.

Materials and Methods

Operative Procedure. 3 adult mongrel dogs (19.5–21 kg) were used. The animals were fasted over night. The animals were anesthetized with pentobarbital with an initial dose of 30 mg/kg body weight followed by additional small doses to maintain anesthesia. The animals were intubated with a cuffed endotracheal tube and ventilated spontaneously. Two venous catheters were inserted for injection and blood sampling. An infusion of up to 1,000 ml isotonic saline was given intravenously during the investigation. The duodenum was opened through a longitudinal incision and the proximal and distal papilla were identified. Polyethylene cannulas were inserted in each of the two ducts, thus separating samples with bile (proximal papilla) and pancreatic juice (distal papilla).

The fluid from the distal and proximal papilla was collected at 15-min intervals up to 180 min after intravenous injection of ^{125}I -trypsin. All samples were stored on ice and analyzed within 48 h.

The animals received 0.5 IE secretin/kg body weight and 0.5 IE cholecystokinin (CCK/PZ)/kg body weight intravenously as a bolus dose 10 min before the injection of 5 μg radiolabelled anionic trypsin (50 μCi). This was followed by intravenous infusion of 0.5 IE secretin and 0.5 IE CCK/PZ/h and kg body weight. Blood samples were drawn at 0, 3 and 15 min and then at 15-min intervals up to 180 min.

The radioactivity of serum, bile, and pancreatic fluid was analyzed in a Gamma spectrometer. Protein-bound radioactivity was measured after trichloroacetic acid (TCA) 15% (w/v) precipitation. Gel filtrations were performed on a Sephadex G-100 column (0.9 $\times$ 50 cm) equilibrated with 0.01 mol/l Tris-HCl buffer containing 0.12 mol/l NaCl and 0.005 mol/l EDTA at pH 7.4. Agarose gel electrophoresis was run according to Johansson [4] in 1% agarose in a barbital buffer (0.075 mol/l) containing 0.002 mol/l calcium lactate at pH 8.6. The anionic canine trypsin was measured using single radial immunodiffusion [5].

Secretin 75 clinical units/ampulla and CCK/PZ 75 Ivy dog units/ampulla were products of GIH-Research Unit, Chemistry Department, Karolinska Institutet, Stockholm, Sweden. Sephadex G-25 and G-100 was obtained from AB Pharmacia Fine Chemicals, Uppsala, Sweden and sodium- ^{125}I from Radiochemical Center, Amersham, England. Anionic canine trypsin was purified as described by Borgström [6]. Antisera against anionic canine trypsin were available at the

laboratory and purified as described by Ohlsson and Tegner [7].

The protein was radiolabelled with ^{125}I in a succinate buffer 0.028 mol/l containing NaCl 0.04 mol/l at pH 5.6 using lactoperoxidase [8]. Free and bound ^{125}I was separated on a Sephadex G-25 column (0.9 $\times$ 10 cm) equilibrated with the same succinate buffer containing 2 g/l bovine serum albumin. The specific activity was approximately 10 $\mu\text{Ci}/\mu\text{g}$ protein. The labelled proteins were used within 1 week and 90–95% of the radioactivity could be precipitated with 15% (w/v) TCA.

Gel filtration on a Sephadex G-100 column of a mixture of labelled trypsin and fresh canine serum disclosed two peaks of radioactivity. One large peak containing 75% of the radioactivity eluting in the void volume with a molecular weight corresponding to α -macroglobulin-trypsin complexes and one smaller peak containing the remaining radioactivity eluting in a larger volume corresponding to the molecular weight of α_1 -antitrypsin-trypsin complexes. No radioactivity eluted in the volume corresponding to the molecular weight of free trypsin showing that all labelled trypsin was active and able to bind to the protease inhibitors of serum.

Results

Serum. Following intravenous injection of anionic ^{125}I -trypsin, there was a similar time-dependent disappearance of TCA-precipitated radioactivity in serum samples from all dogs with a half time of 15 min.

Bile. The total secretion during 3 h from the proximal papilla contained 0.7% (0.1–1.2%) of the intravenously injected ^{125}I . 40–55% of this collected radioactivity was precipitable with TCA (fig. 1). Electrophoretic separation of the samples from the proximal papilla shows only one distinct highly anionic protein band, probably albumin. No distinct cationic bands were seen (fig. 2). Some bile samples with a larger amount of radioactivity was subjected to gel filtration (Sephadex G-

100 column). The TCA-precipitable or protein-bound radioactivity was recovered in one peak eluting in the void volume corresponding to high molecular complexes (molecular weight > 100,000 daltons; fig. 3).

Pancreatic Juice. Agarose gel electrophoresis of the samples recovered from the distal papilla disclosed several distinct protein bands, some with cationic mobility, with a pattern typical for pancreatic juice.

The total 3-hour samples from the distal papilla contained 0.1–0.2% of the intravenously injected radioactivity. 5–27% of the totally collected radioactivity was precipitable with TCA (fig. 1). The amount of radioactivity excreted in the pancreatic juice increased slowly with the time but the ratio between protein-bound and free radioactivity was highest in the earlier samples (table I). The concentration of protein-bound radioactivity in the pancreatic juice samples was, however, too low (< 2,000 cpm/ml) to allow any further characterization of the nature of the protein-bound activity with, for example, gel filtration, preparative electrophoresis or precipitation with specific antisera.

Discussion

In earlier investigations the fate of intravenously injected pancreatic trypsin has been thoroughly studied in dogs [9]. The active enzyme is immediately bound to antiproteases, 80% to the macroglobulin and 20% to the α_1 -antitrypsin [9]. The macroglobulin complexes are then rapidly eliminated from serum by the reticuloendothelial cell system, i.e. the Kupffer cells of the liver [10].

A number of studies have suggested the existence of an enteropancreatic circulation including the absorption of activated pan-

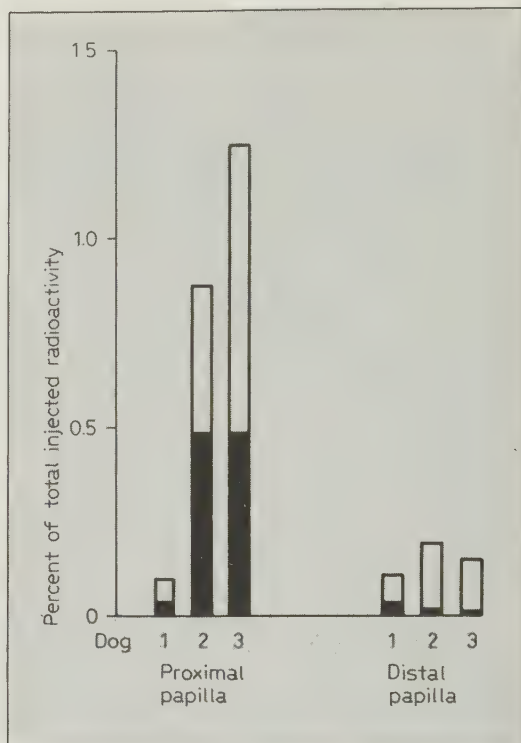


Fig. 1. The collected radioactivity from the proximal and distal papilla during 3 h after intravenous injection of ^{125}I -trypsin. Filled bars illustrate TCA-precipitable radioactivity.

creatic enzymes from the gut to the blood stream, but the subject is very controversial. *Liebow and Rothman* [11] reported such a circulation of heterologous chymotrypsin in the rabbit and it was postulated that circulation of pancreatic enzymes might serve as a 'conservation mechanism'. Regarding amylase, it has been clearly demonstrated that no enteropancreatic circulation exists either in dog [12] or in rat [13]. However, in some reports it has been claimed that an enteropancreatic circulation of trypsin in man ex-

Fig. 2. Agarose gel electrophoresis of the samples collected from the proximal papilla after intravenous injection of ^{125}I -trypsin. Serum sample to the left for reference.

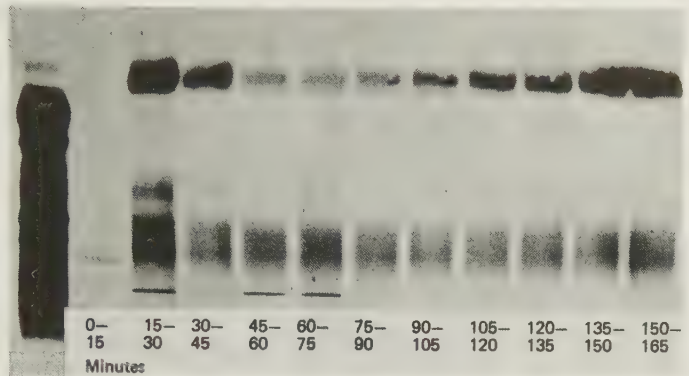


Fig. 3. Gel filtration of a bile sample collected after intravenous injection of ^{125}I -trypsin on a Sephadex G-100 column (0.9×50 cm). Fraction volume 700 μl .

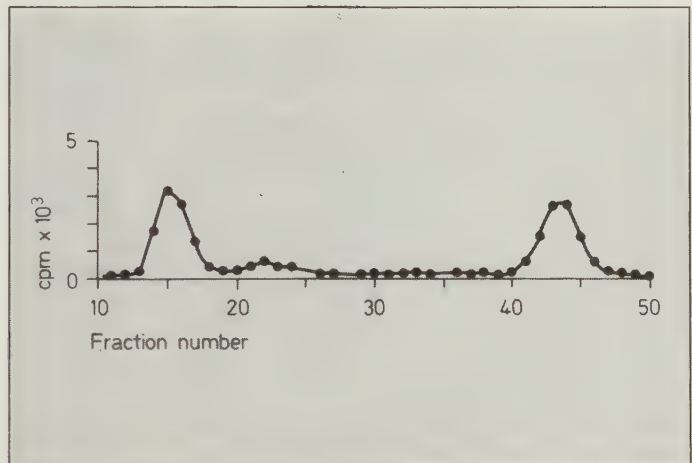


Table I. Analysis of pancreatic juice collected in 15-min fractions after intravenous injection of ^{125}I -trypsin (mean values of 3 dogs)

	Fraction, min											
	0-15	15-30	30-45	45-60	60-75	75-90	90-105	105-120	120-135	135-150	150-165	165-180
Volume, ml	9.3	7.4	7.7	6.7	5.7	5.8	8.7	7.9	5.9	7.0	7.8	9.8
Anionic canine trypsin, mg/ml	0.7	0.6	0.7	0.7	0.7	0.7	0.7	0.7	0.6	0.7	0.4	0.3
cpm/500 μl	360	308	528	1,056	1,211	1,300	1,286	1,449	1,760	1,720	1,865	2,037
TCA-precipitable radioactivity/ total radioactivity	0.25	0.28	0.13	0.11	0.08	0.08	0.05	0.05	0.12	0.06	0.06	0.04

ists [2, 14]. In these experimental studies in man, the amount of given labelled enzymes was so small that characterization of the recovered radioactivity must have been very difficult.

Also in the present study after intravenous injection of homologous canine trypsin, only a very small amount of the injected radioactivity could be recovered in the pancreatic juice and the TCA-precipitable or protein-bound part corresponds to less than 0.05% of the injected ^{125}I . The nature of this protein-bound activity is uncertain. It is, however, hard to understand that these trace amounts of radioactivity can serve as a ground for hypothesis on recirculation of pancreatic enzymes. This matter has recently been thoroughly debated [15, 16]. Even if one accepts the possibility that active pancreatic proteolytic enzymes can cross the intestinal mucosa and be absorbed into the circulation, it is hard to understand how they can escape the binding to the plasma inhibitors and the rapid destruction by the reticuloendothelial cell system of the liver [9, 10]. Further, the whole concept of pancreatic enzymes synthesis with a messenger RNA coding for a pre-pro-enzyme for trypsin [17] is also hard to combine with a recirculation theory for trypsin. The small amount of the TCA-precipitable radioactivity recovered in the bile that had a molecular weight of 100,000 daltons, might represent a spillover of the trypsin-trypsin inhibitor complexes from the reticuloendothelial cell system of the liver [9].

No radioactivity with the molecular weight corresponding to free trypsin was seen in the bile. This result contradicts the finding of recirculation to the bile of small amounts of radiolabelled amylase injected intravenously in dogs [15].

In conclusion, the findings in this study support the standpoints put forward by *Rosenblum* et al. [15] and *Levitt* et al. [12] and argue against a biologically significant enteropancreatic circulation of trypsin.

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References

- 1 Borgström, A.; Genell, S.; Ohlsson, K.: Elevated fecal levels of endogenous pancreatic endopeptidases after antibiotic treatment. *Scand. J. Gastroent.* 12: 525-529 (1977).
- 2 Lake-Bakaar, G.; Rubio, C.E.; McKavanagh, S.; Potter, B.J.; Summerfield, J.A.: Metabolism of ^{125}I -labeled trypsin in man: evidence for recirculation. *Gut* 21: 580-586 (1980).
- 3 Götze, H.; Rothman, S.S.: Enteropancreatic circulation of digestive enzyme as a conservation mechanism. *Nature, Lond.* 257: 607-609 (1975).
- 4 Johansson, B.G.: Agarose gel electrophoresis. *Scand. J. clin. Lab. Invest.* 29: suppl. 127, pp. 7-19 (1972).
- 5 Mancini, G.; Carbonara, A.O.; Heremans, J.F.: Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry* 2: 235-254 (1965).
- 6 Borgström, A.: Purification and N-terminal amino acid sequence determination of anionic and cationic canine trypsinogens. *Hoppe-Seyler's Z. physiol. Chem.* 360: 657-661 (1979).
- 7 Ohlsson, H.; Tegner, H.: Anionic and cationic dog trypsin. Isolation and partial characterization. *Biochim. biophys. Acta* 317: 328-337 (1973).
- 8 Thorell, J.I.; Johansson, B.G.: Enzymatic iodination of polypeptides with ^{125}I to high specific

- activity. *Biochim. biophys. Acta* 251: 363-367 (1971).
- 9 Ohlsson, K.: Interactions in vitro and in vivo between dog trypsin and dog plasma protease inhibitors. *Scand. J. clin. Lab. Invest.* 28: 219-223 (1971).
 - 10 Ohlsson, K.: Elimination of ^{125}I -trypsin- α -macroglobulin complexes from blood by reticuloendothelial cells in dog. *Acta physiol. scand.* 81: 269-272 (1971).
 - 11 Liebow, C.; Rothman, S.S.: Enteropancreatic circulation of digestive enzymes. *Science* 189: 472-474 (1975).
 - 12 Levitt, M.D.; Ellis, C.J.; Murphy, S.M.; Schwartz, M.L.: Study of the possible enteropancreatic circulation of pancreatic amylase in the dog. *Am. J. Physiol.* 241: G54-G58 (1981).
 - 13 Rohr, G.; Kern, H.; Scheele, G.: Enteropancreatic circulation of digestive enzymes does not exist in the rat. *Nature, Lond.* 292: 470-472 (1982).
 - 14 Heinrich, H.C.; Gabbe, E.E.; Brüggemann, J.; Ićagić, F.: Enteropancreatic circulation of trypsin in man. *Klin. Wschr.* 57: 1295-1297 (1979).
 - 15 Rosenblum, J.L.; Raab, B.K.; Alpers, D.H.: Hepatobiliary and pancreatic clearance of circulating pancreatic amylase. *Am. J. Physiol.* 243: G21-G27 (1982).
 - 16 Rothman, S.S.; Grendell, J.H.; Rosenblum, J.L.; Raab, B.K.; Alpers, D.H.; Levitt, M.D.; Rohr, G.; Kern, G.; Scheele, G.: Is there an enteropancreatic circulation of digestive enzyme? Letters to the editor. *Am. J. Physiol.* 244: G101-G106 (1983).
 - 17 Devillers-Thiery, A.; Kindt, T.; Scheele, G.; Blobel, G.: Homology in amino-terminal sequence of precursors to pancreatic secretory proteins. *Proc. natn. Acad. Sci. USA* 72: 5016-5020 (1975).

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Trypsin-Like Immunoreactivity in Human Paneth Cells

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Key Words. Immunohistology · Paneth cells · Trypsin immunoreactivity

Abstract. Human intestinal Paneth cells characterized by their content of lysozyme were shown to contain cationic trypsin immunoreactivity. This trypsin-like immunoreactivity was shown in the Paneth cells at their normal localization at the basis of the crypts of Lieberkühn and also in Paneth cells of metaplastic areas in gastric mucosa. This original finding is a further indication of a resemblance between Paneth and acinar pancreatic cells.

The function of the intestinal Paneth cells originally described by Schwalbe in 1872 and later by Paneth in 1888 is still a matter of considerable controversy. Some properties of the cells appear, however, rather well-settled like their content immunoglobulins and lysozyme and their capability to secrete these proteins into the gut lumen and to eliminate metals from the intestinal contents. The characteristic feature of the cells is the apically located large cytoplasmatic granules and an ultrastructural organisation very similar to pancreatic acinar cells. These and other properties and proposed functions have been well summarized in a recent progress report by *Sandow and Whitehead* [1].

During immunochemical studies of trypsin in human pancreas and gastrointestinal

mucosa, we have found distinctive trypsin immunoreactivity in the Paneth cells of both normal small intestinal mucosa and metaplastic mucosal types in the stomach. Such trypsin-like immunoreactivity seems hitherto to be unknown in the literature on Paneth cells.

Materials and Methods

Gastrointestinal specimens from various types of surgical cases were taken routinely and fixed in 10% buffered formalin and embedded in paraffin. The following types of mucosa were represented: gastric mucosa of normal and intestinal metaplastic type, duodenum and small intestine. The tissue samples were analyzed with light microscopy with hematoxylin-eosin staining. The peroxidase-antiperoxidase (PAP)



Fig. 1. Normal ileal mucosa. The Paneth cells are well demonstrated by their positive immunohistochemical staining for lysozyme (arrows). Rabbit anti-lysozyme serum, 1/500. $\times 22$.



Fig. 2. Normal ileal mucosa. Marked positive staining for trypsin-like immunoreactivity in the Paneth cells (arrows). Rabbit antitrypsin serum, 1/500. $\times 25$.

method according to *Sternberger et al.* [2], with some modifications earlier described [3], was used for localizing trypsin and lysozyme.

An antiserum against human cationic trypsin was produced in rabbits by immunization with pure human cationic trypsin purified according to *Ohlsson and Skude* [4]. Before immunization the trypsin was inactivated with di-iso-propyl-fluorophosphate. The specificity of the antiserum was tested with conventional immunoelectrophoresis against human pancreatic juice and serum. A slight cross-reaction with anionic trypsin/trypsinogen was noted, but the antiserum does not cross-react with any other protein in pancreatic

juice or plasma. A specific rabbit antihuman lysozyme serum was obtained from Prof. *Carl-Bertil Laurell*. The antisera were used in serial dilutions, 1/100, 1/500, and 1/1,000. Controls were performed with each new staining series using the antisera after immunosorbent absorption with the appropriate antigen.

Swine anti-rabbit IgG, PAP, and normal swine serum were obtained from Daco Immunoglobulins, Copenhagen. Swine anti-rabbit IgG and peroxidase complexes were used in the dilution of 1/50, respectively. All immunoreagents were applied for 30 min at room temperature and in a humidified chamber.

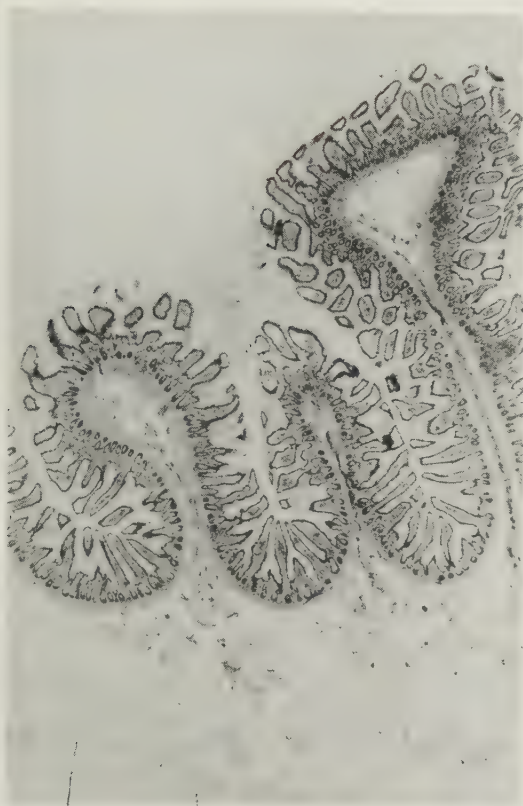


Fig. 3. Normal ileal mucosa. Complete abolishment of trypsin-like immunoreactivity. Immunohistochemical staining using the same antiserum as in figure 2 after trypsin absorption. $\times 23$.

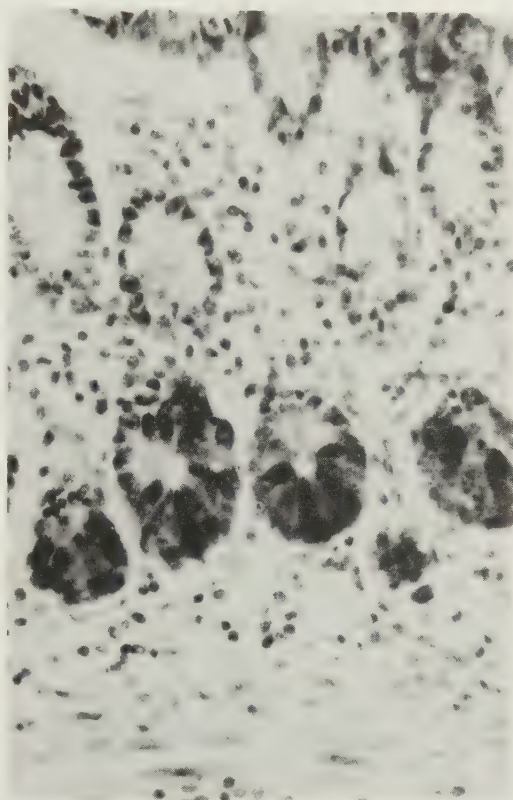


Fig. 4. Normal ileal mucosa. Detail of the basal parts of the crypts of Lieberkühn demonstrating marked trypsin-like immunoreactivity in Paneth cells. Rabbit antitrypsin serum, 1/500. $\times 520$.

Results

We found a marked positive brown staining for trypsin in the Paneth cells of various locations. The Paneth cells were identified on the basis of histologic criteria and by their immunocytochemical staining for lysozyme (fig. 1). Trypsin immunoreactive material was found both in the apical and the basal parts of the cell (fig. 2 and 4). In control sections incubated with the specific antiserum

previously absorbed with trypsin, the positive staining was completely abolished confirming the specificity of the reaction for trypsin (fig. 3).

Duodenal and Small Intestinal Mucosa. Trypsin-like immunoreactivity was found in the Paneth cells at their normal localization at the basis of the crypts of Lieberkühn (fig. 2).

Gastric Mucosa. The normal gastric mucosa did not contain any trypsin-like immu-

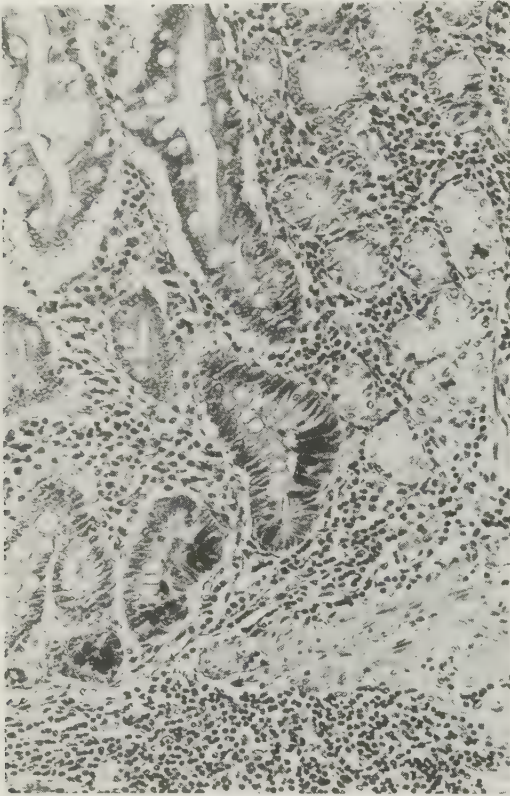


Fig. 5. Gastric mucosa with focal intestinal metaplasia (to the left in the picture). There is a marked trypsin-like immunoreactivity in Paneth cells of the metaplastic mucosa. Rabbit antitrypsin serum, 1/500. $\times 265$.

noreactivity but areas with intestinal metaplasia showed a marked trypsin-like immunoreactivity in the Paneth cells of the metaplastic glands (fig. 5).

Discussion

The ultrastructural resemblance between Paneth cells and the acinar pancreatic cells is a frequent observation in the literature [5]. It

has also been suggested that Paneth cells promote intraluminal digestion of ingested food-stuffs, although the evidence for this has been contradictory and indirect. Our results now show the presence of cationic trypsin immunoreactivity in the Paneth cells further indicating their resemblance with pancreatic acinar cells. In all sections examined the morphological details were excellent. Positive brown staining for trypsin contrasted well with the background staining and was confined to the Paneth cells as identified by their histologic properties and their content of lysozyme. The positive staining was completely abolished in the controls using the specific antiserum previously absorbed with trypsin, further confirming the specificity of the reaction for trypsin.

Furthermore, the rabbit antiserum against trypsin used in this study was recently used in a study showing the presence of trypsin in normal pancreatic tissue and the absence of the same antigen in neoplastic pancreatic tissue [6].

Earlier results indicate that metaplastic Paneth cells in chronic gastritis are identical histochemically and immunocytochemically with normal cells [5]. This is further supported by the present demonstration of an apparently identical content of immunoreactive trypsin in Paneth cells of normal and metaplastic mucosa. The occurrence of Paneth cells and thus also of trypsin immunoreactive material in the gastric mucosa in chronic atrophic gastritis is probably a manifestation of a multipotential ability of the gastrointestinal mucosa to react on various forms of repeated injury.

Trasylol® (aprotinin) is a strong inhibitor of trypsin from all species. In rats fed with Trasylol for a long time an increased number of secretory granules as well as an increasing

granular size of the Paneth cells were shown [7]. Similar effects on the rat pancreas were demonstrated after the administration of soybean trypsin inhibitor [8]. The presence of trypsinogen as a secretory protein in the Paneth cells explains the effects of Trasylol administration. The occurrence of trypsin immunoreactive material in Paneth cells as a result of active production rather than the result of a storage of trypsin-derived material, resorbed from the intestinal content, is also supported by the localization of such cells. Paneth cells of gastric mucosa in chronic atrophic gastritis with intestinal metaplasia contain this trypsin-like immunoreactive material. This is a locale which we do not associate with normal protein digestion and resorption.

The structural resemblance between Paneth cells and the pancreatic acinar cells has been known since long. The present observations further support the view that the Paneth cells are morphologically and functionally, and perhaps also embryologically, related to the pancreatic acinar cells. The Paneth cells may thus, besides other proposed functions, play a role by secreting digestive enzymes like trypsin into the intestinal tract.

Acknowledgements

This investigation was supported by grants from the Swedish Medical Research Council (project No. B84-17X-03910-12C), the Medical Faculty, University of Lund, Thorsten and Elsa Segerfalk's Foundation for Medical Research and Education, Malmö General Hospital Foundation for Cancer, Albert Pahlsson Foundation, Torsten and Ragnar Söderberg Foundation and the Swedish Tobacco Company.

References

- 1 Sandow, M.J.; Whitehead, R.: Progress report of the Paneth cell. *Gut* 20: 420-431 (1979).
- 2 Sternberger, L.A.; Hardy, P.H., Jr.; Cuculis, J.J.; Meyer, H.G.: The unlabelled antibody enzyme method of immunochemistry. Preparations and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. *J. Histochem. Cytochem.* 18: 315-333 (1970).
- 3 Fryksmark, U.; Ohlsson, K.; Polling, Å.; Tegner, H.: Distribution of antileukoprotease in upper respiratory mucosa. *Ann. Otol. Rhinol. Lar.* 91: 268-278 (1982).
- 4 Ohlsson, K.; Skude, G.: Demonstration and semi-quantitative determination of complexes between various proteases and human α_2 -macroglobulin. *Clinica chim. Acta* 66: 1-7 (1976).
- 5 Matsubara, F.: Morphological study of the Paneth cell: Paneth cells in intestinal metaplasia of the stomach and duodenum of man. *Acta path. jap.* 27: 677-695 (1977).
- 6 Marks, W.H.; Ohlsson, K.; Polling, Å.: Immunocytochemical distribution of trypsinogen and pancreatic secretory trypsin inhibitor (PSTI) in normal and neoplastic tissues in man. *Scand. J. Gastroent.* 19: 135-142 (1984).
- 7 Ahonen, A.; Penttilä, A.: Effect of Trasylol on Paneth cells of the mouse. *Experimentia* 31: 577-578 (1975).
- 8 Melmed, R.N.; Turner, R.C.; Holt, S.J.: Intermediate cells of the pancreas. II. Effect of dietary soybean trypsin inhibitor on acinar- β -cell structure and function in the rat. *J. Cell Sci.* 13: 279-295 (1973).

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PANCREATIC ENDOPROTEASES AND PANCREATIC SECRETORY TRYPSIN
INHIBITOR IMMUNOREACTIVITY IN HUMAN PANETH CELLS

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V

Abstract

Normal and metaplastic gastro-intestinal mucosa obtained at surgical resection were studied by light microscopy using the unlabelled antibody enzyme method for immunohistochemical staining of lysozyme, pancreatic endoproteases and pancreatic secretory trypsin inhibitor (PSTI). Paneth cells in the mucosa of normal small intestine, in gastric mucosa with intestinal metaplasia and in colonic metaplastic mucosa were found to contain anionic trypsin, cationic trypsin, lysozyme, and PSTI immunoreactivity but not chymotrypsin and elastase immunoreactivity. Normal gastric and colonic mucosa and some goblet cells in the small intestine showed positive PSTI immunoreactivity but no endoprotease immunoreactivity. The present demonstration of immunoreactive trypsin and immunoreactive PSTI in the Paneth cells, which are of secretory type, probably indicates an important extra-pancreatic source of these proteins rather than a storage of endocytosed material.

Introduction

The Paneth cells originally described by Schwalbe in 1872 (1) and later by Paneth in 1888 (2) are normally found at the base of the crypts of Lieberkühn in the duodenum, jejunum and ileum. Paneth cells are also found in metaplastic areas in gastric or colonic mucosa (3). They are ultrastructurally similar to secretory cells like the pancreatic acinar cells with apically located large cytoplasmatic granules. Among proposed properties and functions are the capability to secrete immunoglobulins and lysozyme into the gut lumen and to eliminate metals from the mucosa into the luminal contents (4).

Using an immunohistochemical technique, we have found a trypsin-like immunoreactivity in the human Paneth cells in the duodenum, the small intestine and in metaplastic areas in the gastric mucosa (5). The purpose of this investigation was to further characterize the trypsin-like immunoreactivity and to determine if the human Paneth cells also contain other pancreatic endoproteases and the pancreatic secretory trypsin

inhibitor (PSTI).

Material and Methods

Tissue samples The material consists of different resection specimens taken during operation from various types of surgical cases. The specimens were fixed in 10% buffered formalin and were imbedded in paraffin. The following types of mucosa are represented: normal mucosa from stomach, duodenum, jejunum, ileum and colon; colonic mucosa from cases with ulcerative colitis containing glands with Paneth cell metaplasia; gastric mucosa of intestinal metaplastic type and in addition colonic adenoma with Paneth cell metaplasia.

Specific material. DEAE-Sephadex, Sepharose 4B and prepacked PD-10 columns were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Trasylol was provided by Bayer AG, West Germany. Swine antirabbit IgG, peroxidase and antiperoxidase (PAP) and normal swine serum were obtained from Daco Immunoglobulins, Copenhagen, Denmark. Di-iso-propyl-fluorophosphate (DFP) was obtained from Sigma Chemicals, St. Louis, USA. Pancreatic juice was obtained from patients having the main pancreatic duct drained after pancreatic surgery. The pancreatic juice was collected in tubes on ice and frozen within 2 hours at -20°C . Human cationic trypsin and human PSTI were purified at the laboratory (6,7).

Antisera: Rabbit antisera against cationic trypsin, chymotrypsin, elastase 2, and PSTI were produced at the laboratory (5,7,8). Specific rabbit antihuman lysozyme serum was obtained from Professor Carl-Bertil Laurell, Malmö, Sweden.

Affinity purification of anionic trypsin on Sepharose-conjugated Trasylol. 150 mg of Trasylol were coupled to 15 g CNBr-activated Sepharose 4B according to the manufacturer's instructions. Pancreatic juice (50 ml) from a patient with high amounts of anionic trypsinogen and only small amounts of cationic trypsinogen was applied to the Sepharose-linked Trasylol column (2.5x10 cm), equilibrated with Tris-HCl buffer

0.01M containing 0.1M NaCl, pH 8. After thorough washing with the starting buffer until absorbance at 280 nm reached zero, the anionic trypsinogen was eluted with 0.01M formic acid containing 0.2M NaCl, pH 3.0. The trypsinogen-containing fractions were desalted immediately on PD-10 columns, equilibrated with 0.001M HCl and lyophilized. The purified anionic trypsinogen showed only one protein band on agarose gel electrophoresis at pH 8.6. After auto-activation this band has a slightly less anionic mobility. The anionic trypsin was inactivated with DFP by adding 10 μ l DFP (1M/l) in iso-propanol to 1 ml of anionic trypsin (1 mg/ml) in HCl 0.001M/l. After the addition of DFP, the pH was adjusted to 7.0 by adding 100 μ l Tris buffer, 1M/l.

Antiserum against human anionic trypsin(ogen) was produced by immunization of rabbits with human anionic trypsin inactivated with DFP and emulsified with Freund's adjuvant. Immuno-electrophoresis against human pancreatic juice before and after activation showed one strong anionic precipitate, corresponding to anionic trypsinogen and trypsin. Sometimes a faint precipitate was seen corresponding to cationic trypsin(ogen), probably indicating a cross reaction between the two tryptsins, as has been reported earlier (9).

Immuno-adsorption of rabbit antisera against human anionic and cationic tryptsins. 8.8 mg DFP-inactivated human cationic trypsin were conjugated to 1 g of Sepharose 4B, and 15 mg DFP-inactivated human anionic trypsin were coupled to 1.5 g of Sepharose 4B, according to the manufacturer's instructions. Two separate columns, 0.9x7 cm and 0.9x11 cm, were equilibrated in a glycine buffer, 0.1M/l, containing NaCl 0.15M/l, EDTA 0.005M/l and NaN_3 0.02% at pH 6.8. Rabbit antiserum against human anionic trypsin (25 ml) was applied to the Sepharose-cationic DFP-trypsin column. The column was then washed with the same glycine buffer. The protein-containing fractions were pooled and applied to the Sepharose-anionic DFP-trypsin column. The column was washed with the same glycine buffer until absorbance at 280 nm was below 0.1. Then the specific antibodies against human anionic trypsin were eluted with a

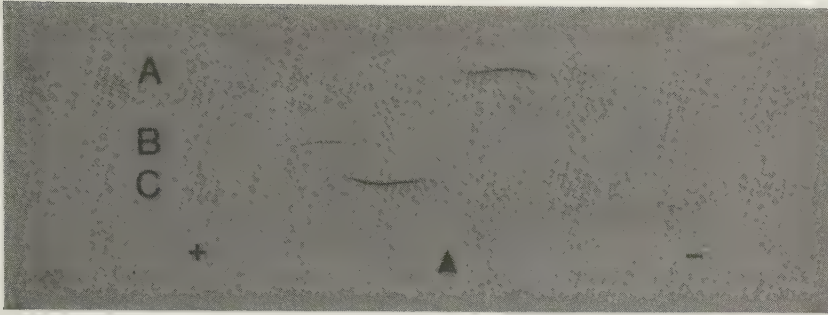


Fig. 1 Precipitation patterns obtained by immunoelectrophoresis of pancreatic juice with A/ rabbit anti-cationic-trypsin serum, B/ rabbit anti-anionic trypsin serum and C/ rabbit anti-PSTI serum. The arrow denotes the application points.

glycine buffer, 0.1M/l, containing NaCl 0.15M/l, NaSCN 3.5M/l, EDTA 0.005M/l and NaN_3 0.02% at pH 7.3. The UV absorption of the effluent was measured and the antibody-containing fractions were pooled and dialysed against H_2O .

The antiserum against human cationic trypsin was processed in the same way; first being filtered through the Sepharose-anionic DFP-trypsin column and the effluent from this column then being applied to the Sepharose-cationic DFP-trypsin column. After washing with the starting buffer to a UV absorption of less than 0.1, the specific antibodies against human cationic trypsin were eluted with the buffer described above. The antibody-containing fractions were pooled and dialysed against H_2O .

The immuno-adsorbed specific rabbit antibodies against human anionic and cationic trypsin were checked by immunoelectrophoresis against human pancreatic juice. The antibodies were used in dilutions made from a stock-solution of 2 mg/ml (UV factor 1.2).

Immunohistochemical methods. The tissue samples were analysed with light microscopy with hematoxylin-eosin staining. The peroxidase-antiperoxidase (PAP) method described by Sternberger et al.(10) with a slight modification earlier described (11) was used for localization of the different pancreatic

Table 1

Pancreatic endoproteases and PSTI immunoreactivity in Paneth cells. Number of investigated specimens with positive immunohistochemical staining.

	Anionic trypsin	Cationic trypsin	PSTI	Chymotrypsin	Elastase
<u>Normal mucosa</u>					
Duodenum	4/4	4/4	4/4	0/4	0/4
Jejunum	4/4	4/4	4/4	0/4	0/4
Ileum	4/4	4/4	4/4	0/4	0/4
<u>Metaplastic mucosa</u>					
Stomach	4/4	4/4	4/4	0/4	0/4
Colon (colitis)	5/6	5/6	5/6	0/6	0/6
Adenoma (Paneth cell metaplasia)	1/1	1/1	1/1	0/1	0/1

endoproteases, PSTI and lysozyme. The antisera were used in serial dilutions 1/100 to 1/2000. Controls were performed with each new staining series using the antisera after immunosorbent absorption with their appropriate antigens and with non-immune rabbit serum. Swine antirabbit IgG and peroxidase complexes were used in the dilutions of 1/50. All reagents were applied for 30 min at room temperature in a humidified chamber.

Results

After immuno-adsorption of the anionic and cationic trypsin antisera, no cross reactivity between them was seen and only one distinct precipitation line was seen on immunoelectrophoresis for each antisera (Fig. 1).

The results of the immunohistochemical staining are given in Table I. The Paneth cells were identified on the basis of histological criteria and by their immunohistochemical staining for lysozyme (Fig. 2). A marked positive brown staining

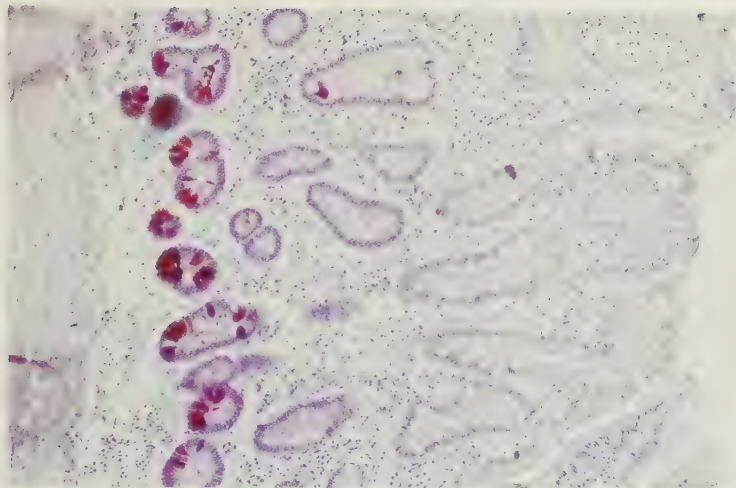


Fig. 4 Gastric mucosa with focal intestinal metaplasia. Anionic trypsin demonstrated within the Paneth cells by immunohistochemical staining. Hematoxylin-eosin. x 160.

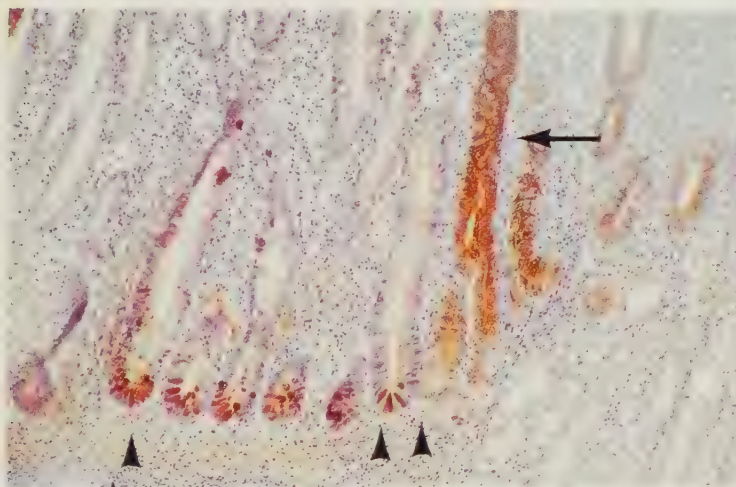


Fig. 5 Gastric mucosa of fundus type with chronic gastritis and marked intestinal metaplasia. PSTI demonstrated within Paneth cells (arrow heads) as well as in the normal glands (arrow) by immunohistochemical staining. Hematoxylin-eosin. x 160.

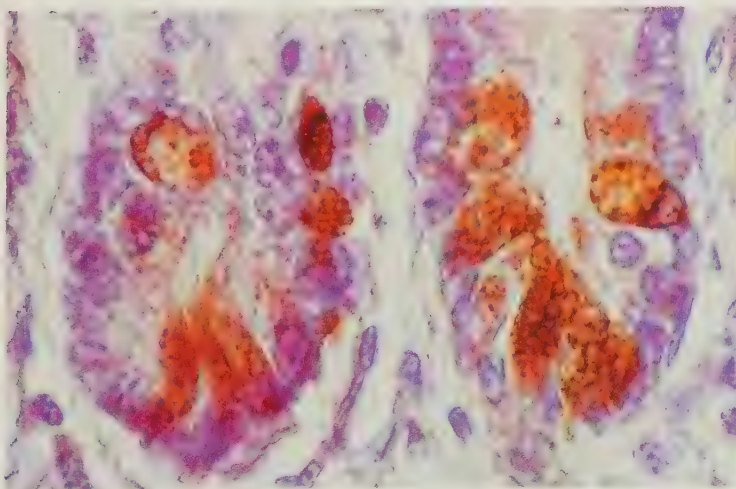


Fig. 9 Normal duodenum mucosa. PSTI demonstrated in the Paneth cells as well as goblet cells by immunohistochemical staining. Hematoxylin-eosin. x 1600.



Fig. 2 (Left) Normal jejunal mucosa. Lysozyme demonstrated within Paneth cells by immunohistochemical staining. x 160.



Fig. 3 (Right) Normal jejunal mucosa. The complete blocking of the staining reaction using absorbed antisera against anionic trypsin. x 160.



Fig. 6 Normal jejunal mucosa. Anionic trypsin demonstrated within the Paneth cells by immunohistochemical staining. x 160.



Fig. 7 Normal jejunal mucosa. Cationic trypsin demonstrated within Paneth cells by immunohistochemical staining. x 160.



Fig. 8 Normal jejunal mucosa. Anionic trypsin demonstrated within Paneth cells by immunohistochemical staining. x 640.

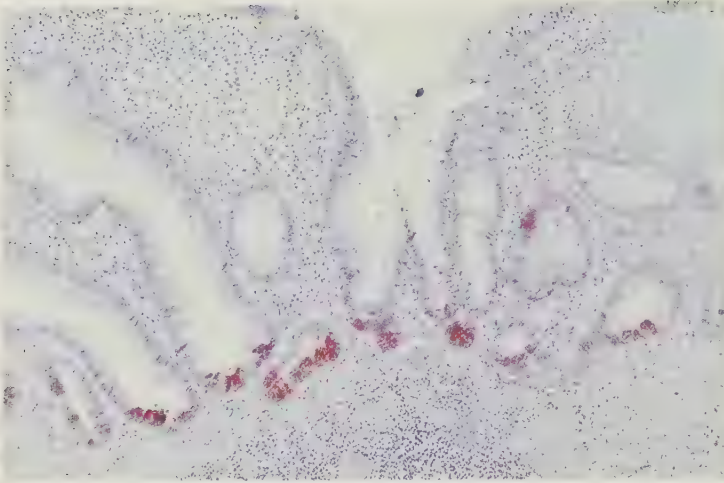


Fig. 10 Colonic mucosa in a case of ulcerative colitis with intestinal metaplasia containing Paneth cells. Anionic trypsin demonstrated within Paneth cells by immunohistochemical staining. Hematoxylin-eosin. x 160.

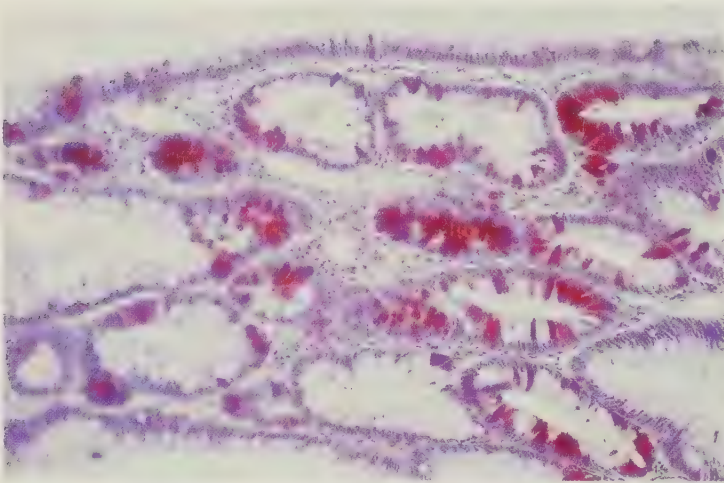


Fig. 11 Colonic adenoma containing Paneth cells. Anionic trypsin demonstrated within Paneth cells by immunohistochemical staining. Hematoxylin-eosin. x 160.

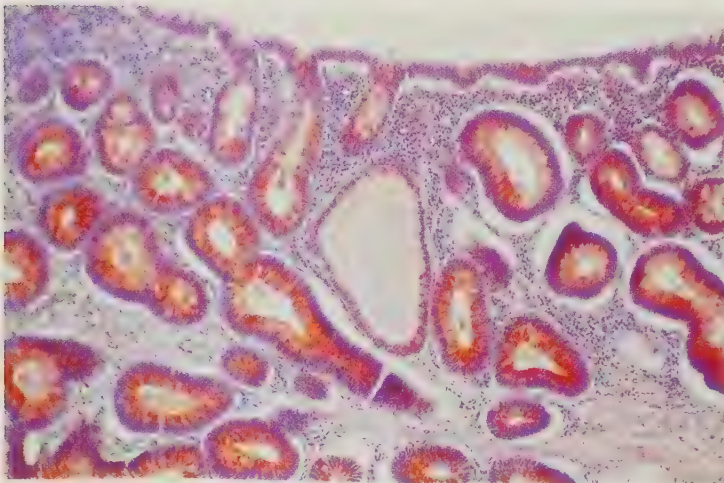


Fig. 12 Colonic adenoma containing Paneth cells. PSTI demonstrated within Paneth cells by immunohistochemical staining. Hematoxylin-eosin. x 160.

for the two trypsins and for PSTI was evident in Paneth cells of various locations. Control sections with normal rabbit sera or antisera previously adsorbed against their appropriate antigens were negative (Fig. 3). No pancreatic chymotrypsin or elastase immunoreactivity could be demonstrated in any control specimen.

Stomach

In normal gastric mucosa no trypsin immunoreactivity was identified. In areas of intestinal metaplasia with Paneth cells, a distinct trypsin immunoreactivity of both anionic and cationic types was identified in the Paneth cells (Fig. 4). The Paneth cells also contained PSTI immunoreactive material (Fig. 5). PSTI immunoreactive material was also seen in some areas of normal mucosa.

Duodenum, jejunum, ileum

In all specimens analysed, a distinct positive anionic and cationic trypsin immunoreactivity was seen in the Paneth cells both in the basal and apical parts of the cells (Fig. 6,7,8). PSTI immunoreactivity was seen in some Paneth cells and also in goblet cells in the basal parts of the glandular crypts of Lieberkühn (Fig. 9).

Colon

In the normal colonic mucosa a positive PSTI immunoreactivity was seen mainly in the basal part of the crypts. In the normal colonic mucosa no Paneth cells were identified and no pancreatic endoprotease immunoreactivity was seen. In metaplastic areas of the mucosa in cases with ulcerative colitis, containing Paneth cells, positive anionic (Fig. 10) and cationic trypsin as well as PSTI immunoreactivity was identified within the cells. In one case of adenoma with Paneth cell metaplasia distinct anionic and cationic trypsin immunoreactivity was identified (Fig. 11). PSTI immunoreactive material was also stored in various parts of the adenoma (Fig.12).

Discussion

In a recent study we reported on trypsin-like immunoreactivity in the human Paneth cells in normal mucosa and in metaplastic areas of gastric mucosa using immunohistochemical methods (5). One purpose of the present study was to elucidate whether both anionic and cationic trypsin immunoreactivity was present in these cells. As the two trypsins show some cross-reactivity, it was necessary to eliminate the cross reacting antibodies from each antiserum. This was accomplished using an immunoadsorption technique with the two isolated antigens bound in a solid phase. Applying the respective specific antibodies, it was possible to show that the trypsin-like immunoreactivity in the Paneth cells is of both anionic and cationic types. The positive brown staining was completely abolished in the controls using the specific antisera previously adsorbed with their appropriate antigens, further confirming the specificity of the reaction for anionic trypsin and cationic trypsin. The Paneth cells of all tissues studied were easily identified according to their histological criteria and by their immunohistochemical staining for lysozyme. In addition to Paneth cells of metaplastic and normal types immunoreactive PSTI could be demonstrated in areas of normal gastric and colonic mucosa and in some goblet cells in the basal parts of the crypts of Lieberkühn in the small intestine. No immunoreactive trypsin was identified in the goblet cells. Thus, immunoreactive trypsin was always accompanied by immunoreactive PSTI fitting the general concepts of Laskowsky (12): tissues containing a protease always also contain the appropriate inhibitor. Further direct isolation and characterization of the demonstrated immunoreactivities will probably clarify the presence of PSTI in some cells without apparent trypsin content.

A frequent observation in the literature is the ultrastructural resemblance between Paneth cells and the acinar pancreatic cells (4). Several findings also point to a functional resemblance. Paneth cells are of secretory type, where pilocarpine has been shown to increase the secretion (13). Trasylol, a potent trypsin inhibitor, increases the granule size in the

Paneth cells (14). Studies in the hamster have shown that pancreatic duct ligation was followed by degeneration of exocrine pancreas and caused a hypertrophy of Paneth and goblet cells (15). The duodenal mucosa in patients suffering from chronic pancreatitis showed a significant increase in the number of Paneth cells (16). These data as well as our demonstration of both trypsin immunoreactive material and PSTI immunoreactive material in the same type of cells argue for a production of trypsin and PSTI in the Paneth cells. The findings of both immunoreactive anionic and cationic trypsin as well as their appropriate inhibitor PSTI in the Paneth cells demonstrated, from a physiological and pathophysiological point of view, a probably important extra-pancreatic source of these enzymes and a further indication of the resemblance between Paneth cells and acinar pancreatic cells.

Acknowledgements

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References

- 1 Schwalbe G. Beiträge zur Kenntnis der Drüsen in der Darmwandungen, in's Besondere der Brunner'schen Drüsen. Arch Mikroskop Anatom 1872; 8:92-140.
- 2 Paneth J. Über die secernierenden Zellen des Dünndarm-Epithels. Arch Mikroskop Anatom 1888; 31:113-191.
- 3 Lewin K. The Paneth cell in disease. Gut 1969; 10:804-811.
- 4 Sandow M, Whitehead R. The Paneth cell. Gut 1979;20: 420-431.
- 5 Bohe M, Borgström A, Lindström C, Ohlsson K. Trypsin-like immunoreactivity in human Paneth cells. Digestion 1984; 30:271-275.

- 6 Ohlsson K, Skude G. Demonstration and semiquantitative determination of complexes between various proteases and human α_2 -macroglobulin. Clin Chim Acta 1976;66:1-7.
- 7 Eddeland A, Ohlsson K. Purification and immunochemical quantitation of human pancreatic secretory trypsin inhibitor. Scand J Clin Lab Invest 1978; 38:261-267.
- 8 Bohe M, Borgström A, Genell S, Ohlsson K. Determination of immunoreactive trypsin, pancreatic elastase and chymotrypsin in extracts of human faeces and ileostomy drainage. Digestion 1983; 27:8-15.
- 9 Feinstein G, Hofstein R, Koifmann J, Sokolovsky M. Human pancreatic proteolytic enzymes and protein inhibitors. Isolation and molecular properties. Eur J Biochem 1974; 43:569-581.
- 10 Sternberger LA, Hardy PH, Cuculis JJ, Meyer HG. The unlabeled antibody enzyme method of immunohistochemistry. Preparations and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. J Histochem Cytochem 1970; 18:315-333.
- 11 Fryksmark U, Ohlsson K, Polling Å, Tegner H. Distribution of antileukoprotease in upper respiratory mucosa. Ann. Otol Rhinol Laryngol 1982; 91:268-278.
- 12 Laskowsky JRM. Résumé in Bayer-Symposium V "Proteinase Inhibitors", pp 679-684, Springer-Verlag, 1974.
- 13 Trier JS, Lorenzsonn V, Groehler K. Pattern of secretion of Paneth cells of the small intestine of mice. Gastroenterology 1967; 53:240-249.
- 14 Ahonen A, Penttilä A. Effect of Trasylol^R on Paneth cells of the mouse. Experientia 1975; 31:577-578.
- 15 Balas D, Senegas-Balas F, Bertrand C, Frexinos J, Ribet A. Effects of pancreatic duct ligation of the hamster intestinal mucosa. Digestion 1980; 20:157-167.
- 16 Senegas-Balas F, Bastie MJ, Balas D, Escourrou J, Bomme-laer G, Bertrand C, Arany Y, Ribet A. Histological variations of the duodenal mucosa in chronic human pancreatitis. Dig Dis Sci 1982; 27:917-922.

IMMUNOREACTIVE PANCREATIC SECRETORY TRYPSIN
INHIBITOR IN SERA FROM PATIENTS WITH ACUTE ULCERATIVE
COLITIS

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Summary

The serum levels of immunoreactive pancreatic secretory trypsin inhibitor (irPSTI) and immunoreactive cationic trypsin (irCT) were measured in 14 patients admitted for severe acute attacks of ulcerative colitis. Patients with acute colitis had significantly higher irPSTI levels in serum (35 $\mu\text{g/l}$) compared with controls (11.5 $\mu\text{g/l}$), while the serum level of irCT was within normal limits. The normal colonic mucosa has recently been shown to contain irPSTI favouring the theory of an extrapancreatic origin of the increased serum levels of irPSTI in patients with severe active ulcerative colitis.

Key words: Colitis, ileostomy content, pancreatic secretory trypsin inhibitor, trypsin.

Introduction

Pancreatic secretory trypsin inhibitor (PSTI), originally isolated from the pancreatic gland (1), has recently also been extracted from other tissues (2) and isolated from urine in patients with gynaecological carcinoma (3).

Increased serum levels of immunoreactive PSTI (irPSTI) are a regular finding in patients with acute pancreatitis (4,5) and have also been reported in patients with carcinoma of nonpancreatic origin (2). The urinary excretion of PSTI has been reported to be elevated in patients with gynaecological carcinoma and bronchopneumonia (6). We have recently demonstrated irPSTI in normal colonic mucosa (7) and the aim of the present study has been to measure the serum level of irPSTI in patients with acute attacks of ulcerative colitis and to compare this with the serum level of immunoreactive cationic trypsin (irCT). The amount of irPSTI normally reaching the large bowel from the small intestine was studied by analysis of irPSTI in ileostomy contents.

Subjects and Methods

Fourteen patients with severe acute attacks of ulcerative colitis according to the criteria of Truelove (8) treated at the Department of Surgery, Malmö General Hospital, Malmö, Sweden, were included in the study. Six were men and 8 women. The median age was 44 years (33-67 years). Serum was obtained the day after admission and stored at -20°C until analysed. The serum creatinine level was normal in all the patients. Serum samples from eight healthy subjects with no signs of gastrointestinal disease were used as controls. A twenty-four-hour collection of ileostomy content was obtained from 5 patients who had an ileostomy. They all had a proctocolectomy performed because of ulcerative colitis 1-12 years before the study.

Five-gram samples from ileostomy contents were homogenized in 25 ml ice cold 0.9% NaCl and extracted for 2 hours at 4°C . The mixtures were centrifuged at $39,000 \times g$ for 30 min. Di-iso-propyl-fluorophosphate (DFP), a serine protease inhibitor, was added to the supernatants to a final concentration of 10 mmol/l. This was followed by an overnight incubation at 4°C . IrCT in serum and irPSTI in serum and extracts from ileostomy contents were measured by radioimmunoassay (4,9). A half ml serum and 50 μl pancreatic juice incubated with 0.02 mg aprotinin (Trasylol^R), respectively were gel-filtrated on G-50 columns (0.9x60 cm), equilibrated and eluted with 0.05 mol Tris-HCl buffer, containing 0.5 mol NaCl, pH 7.4.

DFP was obtained from Merck, Darmstadt, FRG. G-50 from Pharmacia Fine Chemicals, Uppsala, Sweden. Aprotinin (Trasylol^R) was obtained from Bayer AG, West Germany. Pancreatic juice was obtained from patients having their main duct cannulated after pancreatic surgery. Antisera against CT and PSTI were the same as used in earlier studies (4,9).

Statistics: The Wilcoxon rank sum test, two-tailed, was used. p-Values less than 0.05 were regarded as significant.

Table I

Immunoreactive Pancreatic Secretory Trypsin Inhibitor (irPSTI) and Cationic Trypsin (irCT) in Serum Samples from Patients with Acute Colitis and Healthy Subjects Expressed as $\mu\text{g/l}$. Median value and range are given.

	N	irPSTI	irCT	Ratio irPSTI/irCT
Acute colitis	14	35(6.3-105)	19.2(9.6-50)	1.1(0.1-10.0)
		p<0.05	N.S	p<0.05
Normal subjects	8	11.5(5.6-18)	20.5(10.7-27.3)	0.4(0.29-0.65)

Results and Discussion

Patients with severe acute attacks of ulcerative colitis had a significantly increased irPSTI level in sera compared with controls, while the level of irCT was within normal limits (Table I). Gel-filtration and analyses of patients sera showed a homogenous irPSTI peak corresponding to the molecular size of PSTI corroborating the specificity of the assay in the present analyses. The reason for the increased level of irPSTI in acute colitis is so far not clear. Reabsorption of PSTI of pancreatic origin through the damaged colonic mucosa seems unlikely, since normally only 1.8-9.3 $\mu\text{g/day}$ reach the colon according to the extractable amount from ileostomy contents.

A reduced elimination through decreased glomerular filtration as in uremic patients (4) does not seem probable either, as all patients had normal serum creatinine levels. Increased leakage from the pancreatic gland due to subclinical pancreatitis could also give increased serum levels of irPSTI, but the concomitant normal irCT levels strongly argue against this.

The ratio between PSTI and trypsinogen in pancreatic juice is fairly constant, between 0.02 and 0.03, even after stimulation

of the pancreatic gland (10). The ratio in normal serum is about 15 times higher (0.4), and is now found to be further increased in patients with acute colitis (1.1) (Table I). The difference in ratio between pancreatic juice and serum from normal subjects and patients with acute colitis cannot be explained by different elimination rates, as PSTI is eliminated faster than trypsin or trypsinogen from the circulation (11,12,13). These findings suggest an additional extra-pancreatic origin of irPSTI in serum. As irPSTI, but not irCT, recently has been demonstrated in the normal colonic mucosa, this might be the origin of the increased serum level of irPSTI in patients with severe acute colitis (7).

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Literature

1. Kazal, L.A., Spicer, D.S. & Brahinsky R.A. (1948) J. Am. Chem. Soc. 70, 3034-3040.
2. Matsuda, K., Ogowa, M., Murata A., Kitahara, T. & Kosaki, G. (1983) Res. Commun. Chem. Path. Pharm. 40, 301-305.
3. Huhtala, M.-L., Pesonen, K., Kalkkinen, N. & Stenman, U.-H. (1982) J. Biol. Chem. 257, 13713-13716.
4. Eddeland, A. & Ohlsson, K. (1978) Hoppe-Seyler's Z. Physiol. Chem. 359, 671-675.
5. Otsuki, M., Oka, T., Suehiro, I., Okabayashi, Y., Ohki, A., Yuu, H. & Baba, S. (1984) Clin. Chim. Acta 142, 231-240.
6. Huhtala, M.-J., Kahanpää, K., Seppälä, M., Halila, H. & Stenman, U.-H. (1983) Int. J. Cancer 31, 711-714.
7. Bohe, M., Borgström, A., Lindström, C. & Ohlsson, K. (1986) J. Clin. Pathol. In press.
8. Truelove, S.C. & Witts, L.J. (1955) Br. Med. J. 2, 1041-1048.
9. Borgström, A. & Ohlsson, K. (1976) Scand. J. Clin. Lab. Invest. 36, 809-814.
10. Eddeland, A. & Wehlin, L. (1978) Hoppe-Seyler's Z. Physiol. Chem. 359, 1653-1658.
11. Marks, W.H. & Ohlsson, K. (1983) Scand. J. Gastroent. 18, 955-959.
12. Ohlsson, K. & Laurell, C.-B. (1976) Clin. Sci. Mol. Med. 51, 87-92.
13. Borgström, A. (1981) Scand. J. Gastroent. 16, 281-287.

PANCREATIC AND GRANULOCYTIC ENDOPROTEASES IN FAECAL
EXTRACTS FROM PATIENTS WITH ACTIVE ULCERATIVE
COLITIS

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Summary

Faecal samples from sixteen patients with acute attacks of ulcerative colitis, seven with quiescent disease and eight healthy subjects were studied regarding extractable amounts of casein digestion capacity, immunoreactive anionic trypsin, cationic trypsin, chymotrypsin, pancreatic elastase and granulocytic elastase. Patients with acute attacks of colitis had significantly higher levels of casein digestion, pancreatic elastase and granulocytic elastase in faecal samples compared with patients with quiescent disease and controls. The non-specific proteolytic activity in faecal extracts from patients with acute colitis was mainly due to the pancreatic proteases: anionic elastase, cationic elastase, anionic trypsin and the granulocytic proteases: elastase and neutral protease. These active proteases may cause further destruction of the already damaged mucosa found in patients with severe ulcerative colitis.

Introduction

Ulcerative colitis is a mucosal disease. The aetiology is not known and many theories have been suggested (1). In active disease ulceration, crypt abscesses and an intense granulocyte infiltration are found in the colonic mucosa (2). ^{111}In granulocyte scanning and faecal ^{111}In granulocyte excretion have shown an uptake in the colonic wall and an excretion of half of the injected labelled granulocytes during a four-day period in patients with acute attacks of ulcerative colitis (3). Thus not only pancreatic and bacterial proteolytic enzymes, normally present in the faeces, but also granulocytic proteolytic enzymes can influence the destruction of the colonic mucosa in patients with acute colitis.

The aim of the present study has been to measure and characterize the extractable proteolytic activity in stool samples from patients with acute colitis.

Subjects, material and methods

Subjects: Twenty-three patients with ulcerative colitis, according to the findings on double barium enema or colonoscopy with biopsies, took part in the study. They were treated at the Department of Surgery, Malmö General Hospital, Malmö, during a 4-year period, 1981-1984. Fifteen were men and 8 women. The median age was 44 years (range 33-67 years). Sixteen patients had severe attacks of colitis according to the criteria of Truelove et al. (4) and were hospitalized. Seven patients had quiescent disease and were out-patients. Eight healthy subjects, aged 25-50 years, were used as controls. None of the subjects had taken antibiotics the year before the study.

Material: Agarose, batch no. 50013, was purchased from FMC, USA, Sephadex G-200 from AB Pharmacia Fine Chemicals, Uppsala, Sweden, casein from E. Merck, Darmstadt, GFR and di-iso-propyl-fluorophosphate (DFP) from Sigma Chemicals, St. Louis, USA. Human α_1 -proteinase inhibitor (α_1 -PI) was available at the laboratory (5).

Antisera: Monospecific polyclonal antisera against the human pancreatic endoproteases; cationic trypsin (6), anionic trypsin (7), chymotrypsin (8), cationic elastase (9), and the granulocyte proteases: elastase (10) and neutral protease (11) were produced at the laboratory. Anionic elastase was isolated from pancreatic juice in principle as earlier described (12). A monospecific polyclonal antiserum against anionic elastase was produced by immunization of rabbits with DFP inactivated anionic elastase (6). Monospecific polyclonal antisera against α_2 -macroglobulin (α_2 -M) and α_1 -PI were gifts from professor Carl-Bertil Laurell, Malmö, Sweden.

Methods: Faecal samples were collected from patients with acute colitis the day after admission. Within 4 hours after collection, two grams of each stool sample were homogenized in 10 ml 0.9 % NaCl and extracted for 2 hours at 4°C. The mixtures were centrifuged at 39,000 x g for 30 minutes at 4°C. The

supernatant fluids were separated and either analysed immediately or frozen at -20°C until analysed. Two ml of each supernatant were incubated with DFP to a final concentration of 10 mmol/l as described earlier (8).

Cationic trypsin (6), anionic trypsin (13), chymotrypsin (14) pancreatic cationic elastase (9) and granulocyte elastase (15) were measured in the DFP-treated samples with radioimmunoassay. α_1 -PI and α_2 -M were quantified with electroimmunoassay (16).

Non-specific proteolytic activity was analysed on casein agarose plates containing 50 mg casein in 25 ml 1% agarose in diethyl barbiturate buffer 75 mmol/l, pH 7.4 containing calcium lactate 2 mmol/l. Aliquots (10 μl) of samples were added to the wells punched in the gel. The plates were incubated at 37°C for 24 hours. They were fixed in picric acid, pressed, dried and stained with Comassie Blue. The proteolytic activity was estimated from the size of the lysis zones.

Inhibition assay: Faecal extracts (100 μl) were incubated with α_1 -PI (0-200 μg in 100 μl 0.05M Tris HCl Buffer containing 0.15M NaCl, pH 7.4) for 1 hour at 20°C . The residual non-specific proteolytic activity was measured on casein agarose gels.

Immunochemical identification of active proteases in faecal extracts utilizing the ability of α_1 -PI to form complexes with active proteases : Faecal extracts (100 μl) were analysed before and after the addition of α_1 -PI (in an amount giving total inhibition of the proteolytic activity of the respective extracts) with crossed immunoelectrophoresis using antisera against the different pancreatic and granulocytic proteases and against α_1 -PI (17). The lower limit with this method was 5 μg active protease/ml.

Gel-filtration: Chromatographic characterization of the different immunoreactive (i.r.) endoproteases, non-specific proteolytic activity, α_1 -PI and α_2 -M was performed on five

samples (3ml) on a Sephadex G-200 column (1.6x120 cm), equilibrated and eluted with 0.05M Tris HCl buffer, containing 0.5M NaCl, pH 7.4.

Statistics: The Wilcoxon rank sum test (two-tailed) was used and p-values less than 0.05 were considered significant.

Result

Proteolytic activity of faecal extracts

Patients with acute colitis had a significantly higher non-specific proteolytic activity corresponding to 0.15 g casein digested/g faeces compared with normal subjects or patients with quiescent disease where no proteolytic activity was detected (Table I). The DFP-inactivated samples contained no proteolytic activity. α_1 -PI in a median concentration of 1.1 mg/g faeces (0-4.4 mg/g faeces) inhibited all proteolytic activity.

Analyses of faecal extracts by crossed immunoelectrophoresis with antiserum against anionic trypsin showed the presence of one component with the electrophoretic mobility of the native enzyme (Fig. 1A). The addition of α_1 -PI to the faecal extracts caused the disappearance of this trypsin component and a new precipitate of i.r. anionic trypsin obtained in the α_1 -region (Fig. 1B). This component was also precipitated by antibodies against α_1 -PI (Fig. 1C). It thus consisted of complexes between anionic trypsin and α_1 -PI. This means that the faecal extracts also contained free, active anionic trypsin bound by the α_1 -PI added in stable complexes. Identical analyses with antiserum against anionic (results not shown) and cationic pancreatic elastase (Fig. 2A,2B) demonstrated the presence of active anionic and cationic pancreatic elastase in faecal extracts. No active cationic trypsin or chymotrypsin could be demonstrated with this method.

Table I

Proteolytic activity and immunoreactive pancreatic proteases and granulocytic elastase in faecal extracts in patients with acute attacks or quiescent colitis and healthy subjects measured per g wet weight faeces. Median and range are given. (N.d. = not detected).

	Patients with acute colitis	Patients with quiescent colitis	Healthy subjects
Number of patients	16	7	8
Non-specific proteolytic activity g casein digested/g	0.15 ^{*a,b} (N.d.-0.43)	N.d.	N.d.
Cationic trypsin µg/g	2.5 (1.0-21.0)	5.0 (1.0-82.5)	2.4 (0.5-4.4)
Anionic trypsin µg/g	10.0 ^{*b} (1.0-50.0)	10.0 ^{*c} (2.0-40.0)	1.0 (0.5-2.0)
Chymotrypsin µg/g	1.6 (N.d.-8.5)	2.0 (0.5-5.0)	1.5 (0.5-4.2)
Cationic elastase µg/g	13.0 ^{**a,b} (1.5-180.0)	2.5 (0.15-4.0)	0.6 (0.2-3.9)
Granulocytic elastase µg/g	26.0 ^{**a,b} (20.0-80.0)	0.2 ^{**c} (0.05-0.35)	0.005 (N.d.-0.03)

*= $p < 0.05$, **= $p < 0.01$, a=patients with acute colitis compared with patients with quiescent colitis, b=patients with acute colitis compared with healthy subjects and c=patients with quiescent colitis compared with healthy subjects.

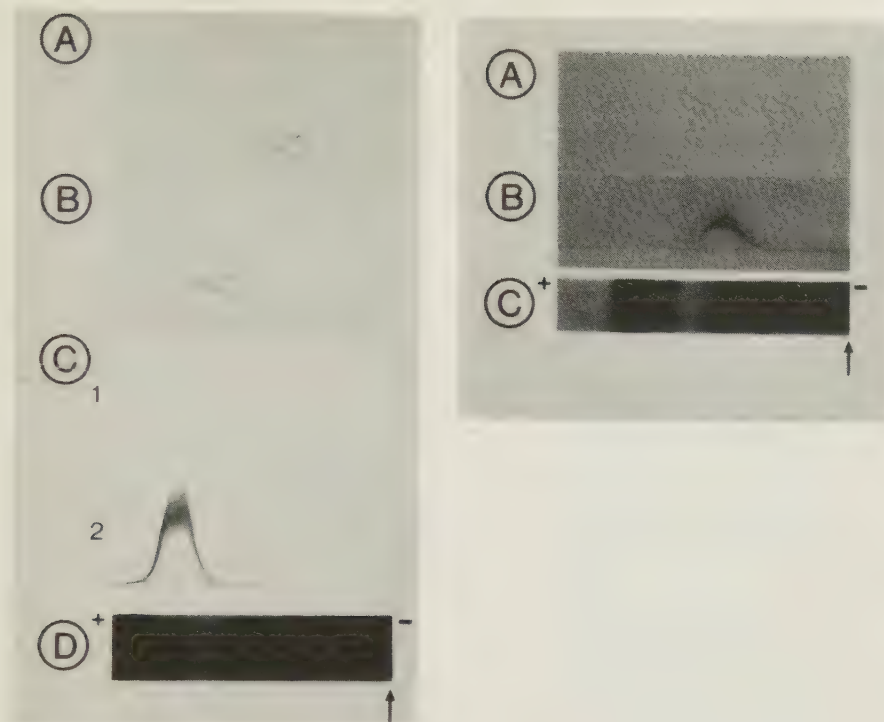


Fig. 1 (Left) Precipitation pattern obtained on crossed immunoelectrophoresis of faecal extracts before (A) and after (B,C) the addition of native α_1 -PI. A,B,C1 contain antiserum against human anionic trypsin and C2 contains antiserum against α_1 -PI. Thus, A shows the precipitation pattern of native anionic trypsin and B trypsin in complex with α_1 -PI, as this peak is also precipitated by antiserum against α_1 -PI. D. Electrophoresis of normal serum. Arrow indicates the application point.

Fig. 2 (Right) Precipitation pattern of faecal extract before (A) and after (B) the addition of α_1 -PI, using antiserum against human pancreatic cationic elastase. The active cationic elastase in faecal extracts formed complexes with α_1 -PI with anionic mobility. C. Electrophoresis of normal serum. Arrow indicates the application point.

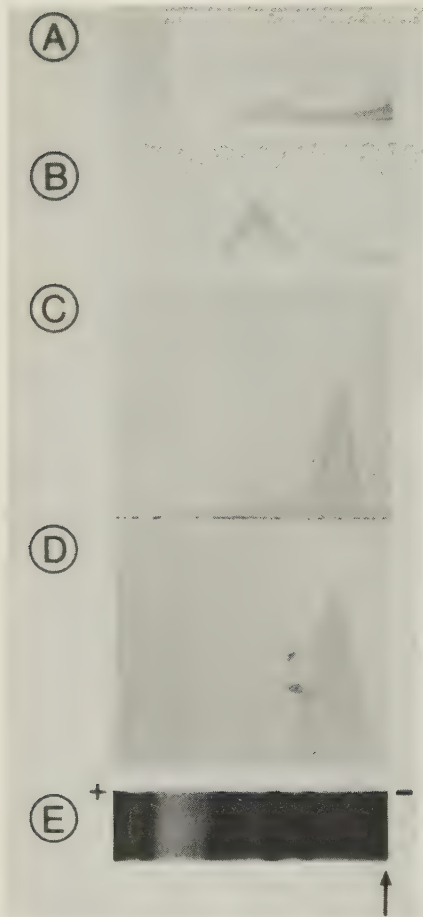


Fig. 3 Precipitation pattern of faecal extracts before (A,C) and after (B,D) the addition of native α_1 -PI. A,B contain antisera against neutral protease and C,D contain antiserum against granulocytic elastase. After the addition of α_1 -PI the precipitates increased showing that the faecal extracts contain both active granulocyte elastase and neutral protease and granulocytic elastase and neutral protease in complex with α_1 -PI. E. Electrophoresis of normal serum. The arrow indicates the application point.

Corresponding analyses utilizing antisera against granulocytic proteases; elastase and neutral protease, showed the presence of small amounts of complexes between these enzymes and α_1 -PI in the faecal extracts (Fig. 3A,3C). The addition of α_1 -PI caused a considerable increase in the precipitate peaks obtained on crossed immunoelectrophoresis utilizing the corresponding antisera (3B,3D). Thus, both enzymes were also present in a free, active form in the faecal extracts.

Radioimmunoassay and gel-filtration studies of i.r. pancreatic and granulocytic endoproteases

The extractable amount of the different i.r. pancreatic endoproteases and i.r. granulocyte elastase is shown in Table I. Patients with acute attacks of colitis had a higher level of

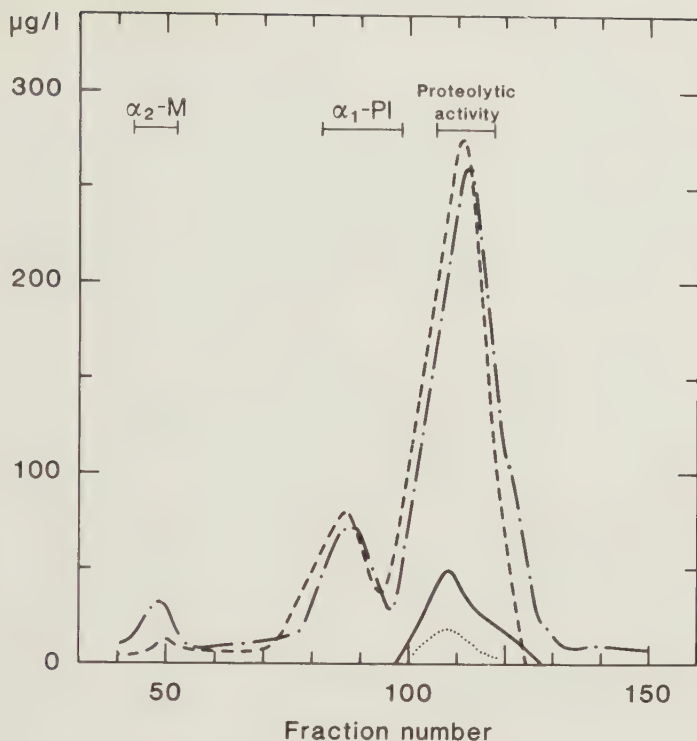


Fig. 4 Chromatographic characterization of immunoreactive (i.r.) pancreatic cationic elastase (--) , i.r. granulocyte elastase (-.-) , i.r. cationic trypsin (...) and i.r. anionic trypsin (—) by gel-filtration on a Sephadex G-200 column (1.6x120 cm) of 3 ml faecal extract. The elution volumes for α_2 -M, α_1 -PI and non-specific proteolytic activity are given as reference.

i.r. pancreatic elastase ($p < 0.01$) and i.r. granulocyte elastase ($p < 0.01$) than patients with quiescent disease and normal subjects.

After chromatographic separation both pancreatic and granulocyte elastase eluted in two volumes, one corresponding to the native protease and one corresponding to elastase in complex with α_1 -PI (Fig. 4). In most samples the levels of cationic trypsin and chymotrypsin were too low to be characterized with gel-filtration. Anionic trypsin eluted in a volume corresponding to the native enzyme and so did the i.r. cationic trypsin in those samples where the analysis was possible.

Discussion

Stool samples from patients with acute attacks of colitis contained significantly higher levels of extractable proteolytic activity compared with normal subjects and patients with quiescent disease. The characterization of the different proteases responsible for the proteolytic activity with enzymatic methods using low-molecular-weight substrates is difficult as these substrates are not specific and some bacteria also produce trypsin and chymotrypsin-like enzymes (18,19,20). We found, however, that DFP, a serine protease inhibitor, inhibited all the proteolytic activity, demonstrating that the proteolysis was due to serine proteases. Furthermore, the plasma protease inhibitor α_1 -PI was also shown to inhibit all proteolytic activity by forming complexes with the active proteases in faecal samples. The complexes formed were further analysed using crossed immunoelectrophoresis and with this method 5 different active proteases were identified, i.e. the pancreatic proteases anionic trypsin, anionic elastase, cationic elastase and the granulocytic proteases elastase and neutral protease. Thus the increased levels of the different i.r. proteases represented also active proteases probably according for the major part of the proteolytic activity present.

In earlier gel filtration studies of faecal extracts and ileostomy contents from healthy subjects pancreatic endoproteases eluted only in one volume corresponding to the native protein (8). In this study, regarding patients with acute colitis, pancreatic elastase and also granulocyte elastase eluted in two volumes, one corresponding to the protease in complex with α_1 -PI and the other major component to the native protease. This finding is in accordance with earlier studies on the plasma protease inhibitors in faecal extracts from patients with ulcerative colitis, where the major part of the protease inhibitors was inactive and unable to bind proteases and a minor part was in complex with the granulocyte proteases elastase and neutral protease (21).

The identification of large amounts of granulocyte proteases

in faecal extracts from patients with acute colitis is in accordance with studies using labelled granulocytes injected into patients with acute colitis where 40 % of the injected radioactivity was recovered in stools during a 4-day period (3). Thus large amounts of granulocytes are not only reaching the colon but also releasing at least part of their protease content, probably accounting for the major part of the proteolytic activity present.

Normally only small amounts of pancreatic endoproteases are extractable from faeces (8). Most of the pancreatic endoproteases reaching the colon are degraded by the bacterial flora (22). Antibiotic treatment gives higher extractable levels of the different pancreatic endoproteases in faecal samples (8,23). In this study none of the patients had taken any antibiotics during the last year and antibiotic treatment could thus not be the cause of the elevated concentration of pancreatic proteases found in faeces from patients with acute colitis.

An increased level of the different pancreatic proteases could be due to an increased rate of propulsion. The ratio between the different extractable pancreatic proteases was however altered. The levels of cationic trypsin and chymotrypsin were the same as in faeces from healthy subjects while the levels of i.r. pancreatic cationic elastase and anionic trypsin were increased. An altered bacterial flora in patients with colitis and a changed degradation in the colon might perhaps explain this divergence.

The genesis of the mucosal damage found in patients with ulcerative colitis is not known. However an already damaged mucosa and submucosa could be further attacked by proteolytic enzymes of granulocytic, pancreatic and bacterial origin. Granulocyte elastase alone has the capacity to degrade all components of connective tissue and to activate or degrade important factors of the cascade system (24). Rats, cats and dogs subjected to haemorrhagic shock showed pronounced mucosal damage (25,26). The damage to the mucosa was less pronounced after inhibition

of the pancreatic proteases using ovomucoid (27), soybean trypsin inhibitor (26) or previous ligation of the pancreatic duct (28).

In conclusion this study has shown that faecal samples from patients with acute colitis have a considerable proteolytic activity characterized by an elevated level of extractable pancreatic elastase, anionic trypsin as well as granulocytic elastase and neutral protease. The presence of active proteases in the faecal extracts from patients with active ulcerative colitis probably reflects a local protease-anti-protease imbalance in the damaged mucosa-submucosa. Local treatment with an appropriate protease inhibitor thus seems worthwhile as a therapeutic attempt to reduce tissue damage.

Acknowledgements

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References

1. Kirsner JB, Shorter RG. Recent developments in non-specific inflammatory bowel disease. *N Engl Med* 1982; **306**: 837-848.
2. Morson BC, Dawson IMP. Ulcerative colitis. In: *Gastrointestinal pathology*, ed 2, Blackwell Scientific Publications, London 1979 pp 523-561.
3. Saverymuttu SH, Hodgson HJF, Chadwick VS. Comparison of fecal granulocyte excretion in ulcerative colitis and Crohn's colitis. *Dig Dis Sci* 1984; **9**: 1000-1004.
4. Truelove SC, Witts LJ. Cortisone in ulcerative colitis. Final report on a therapeutic trial. *Br Med J* 1955; **2**: 1041-1048.
5. Laurell CB, Pierce J, Persson U, Thulin E. Purification of α_1 -antitrypsin from plasma through thiol-disulfide interchange. *Eur J Biochem* 1975; **57**: 107-113.
6. Borgström A, Ohlsson K. Radioimmunological determination and characterization of cathodal trypsin-like immunoreactivity in normal human plasma. *Scand J Clin Lab Invest* 1976; **36**: 809-814.
7. Bohe M, Borgström A, Lindström C, Ohlsson, K. Pan-creatic endoproteases and pancreatic secretory trypsin inhibitor immunoreactivity in human Paneth cells. *J Clin Pathol*. In press.
8. Bohe M, Borgström A, Genell S, Ohlsson K. Determination of immunoreactive trypsin, pancreatic elastase and chymotrypsin in extracts of human feces and ileostomy drainage. *Digestion* 1983; **27**: 8-15.
9. Borgström A, Kukora J, Ohlsson K. Studies on immunoreactive pancreatic elastase 2 in human serum. *Hoppe-Seyler's Z Physiol Chem* 1980; **361**: 633-640.
10. Ohlsson K, Olsson I. The neutral proteases of human granulocytes. Isolation and partial characterization of granulocyte elastases. *Eur J Biochem* 1974; **42**: 519-527.

11. Ohlsson K, Olsson I. The neutral proteases of human granulocytes. Isolation and partial characterization of two granulocyte collagenases. *Eur J Biochem* 1973; **36**: 473-481.
12. Largman C, Brodrick JW, Geokas MC. Purification and characterization of two human pancreatic elastases. *Biochemistry* 1976; **15**: 2491-2500.
13. Largman C, Brodrick JW, Geokas MC, Johnson JH. Demonstration of human pancreatic anionic trypsinogen in normal serum by radioimmunoassay. *Biochim Biophys Acta* 1978; **543**: 450-454.
14. Geokas M, Largman C, Brodrick J, Johnson J, Fasett M. Immunoreactive forms of human pancreatic chymotrypsin in normal plasma. *J Biol Chem* 1979; **254**: 2775-2781.
15. Ohlsson K, Olsson A-S. Immunoreactive granulocyte elastase in human serum. *Hoppe-Seyler's Z Physiol Chem* 1978; **359**: 1531-1539.
16. Laurell C-B. Electroimmunoassay. *Scand J Clin Lab Invest* 1972; **29**, suppl 124: 21-37.
17. Ganrot PO. Crossed immunoelectrophoresis. *Scand J Clin Lab Invest* 1972; **29** suppl 124: 39-47.
18. Pacaud M, Richaud C. Protease II from *Escherichia coli*. Purification and characterization. *J Biol Chem* 1975; **250**: 7771-7779.
19. Pacaud M. Purification of protease II from *Escherichia coli* by affinity chromatography and separation of two enzyme species from cells harvested at late log phase. *Eur J Biochem* 1976; **64**: 199-204.
20. Pacaud M, Sibilli L, Le Bras G. Protease I from *Escherichia coli*. Some physicochemical properties and substrate specificity. *Eur J Biochem* 1976; **69**: 141-51.
21. Bohe M, Genell S, Ohlsson K. Protease inhibitors in plasma and faecal extracts from patients with active inflammatory bowel disease. *Scand J Gastroenterol*. In press.
22. Genell S, Gustafsson BE, Ohlsson K. Immunochemical quantitation of pancreatic endopeptidases in the intestinal contents of germfree and conventional rats. *Scand J Gastroenterol* 1977; **12**: 811-820.

23. Borgström A, Genell S, Ohlsson K. Elevated fecal levels of endogenous pancreatic endopeptidases after antibiotic treatment. *Scand J Gastroenterol* 1977; **712**: 525-529.
24. Ohlsson K. Granulocyte proteases and their inhibitors in inflammation; basic concepts and clinical implications. In: *Inflammation. Basic mechanics, tissue injuring principles, and clinical models*. Venge P. Lindbom A (eds), Almquist & Wiksell International, Stockholm, Sweden pp 379-393
25. Bounous G. Role of the intestinal contents in the pathophysiology of acute intestinal ischemia. *Am J Surg* 1967; **114**: 368-375.
26. Parks DA, Granger DN, Bulkley GB, Shah AK. Soybean trypsin inhibitor attenuates ischemic injury to the feline small intestine. *Gastroenterology* 1985; **89**: 6-12.
27. Bounous G, Proulx J, Konok G, Wollin A. The role of bile and pancreatic proteases in the pathogenesis of ischemic enteropathy. *Int J Clin Pharm Biopharm* 1979; **17**: 317-323.
28. Bounous G, Brown RA, Mulder DS, Hampson LG, Gurd FN. Abolition of 'tryptic enteritis' in the shocked dog. *Arch Surg* 1965; **91**: 371-375.



PROTEASE INHIBITORS IN PLASMA AND FAECAL EXTRACTS FROM
PATIENTS WITH ACTIVE INFLAMMATORY BOWEL DISEASE

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Abstract

The level and functional activity of the major protease inhibitors in plasma and faecal extracts were analysed in twenty-six consecutive patients admitted with their first attack of acute severe colitis. The patients were retrospectively divided into two groups: one with total colitis and another with distal colitis. The patients with total colitis had a significantly lower α_2 -macroglobulin level in plasma than normal individuals and patients with distal disease, while no difference in the levels of α_1 -protease inhibitor, antichymotrypsin, antithrombin III and α_2 -antiplasmin were noted between the two groups. The protease-inhibiting capacity was saturated and free proteolytic activity was present in the faecal extracts. Complex formation was demonstrated in the extracts between leucocyte proteases and the antiproteases α_1 -protease inhibitor and α_2 -macroglobulin. It is concluded that the low plasma level of α_2 -macroglobulin in patients with severe total colitis is mainly due to a consumption caused by complex formation with proteases as earlier demonstrated in patients with acute pancreatitis and sepsis.

Introduction

During acute attacks of inflammatory bowel disease there are marked alterations in the concentration of several serum proteins (1,2). The serum levels of the acute-phase proteins generally increase while the levels of other proteins like albumin decrease, probably as a result of losses through the inflamed intestinal wall (3). An unexplained, low serum level of one of the major serum protease inhibitors, α_2 -macroglobulin, has been reported (2,3,4).

The severe inflammatory processes in the bowel wall are accompanied by pronounced tissue destruction, in which proteases might be involved. A local release of large amounts of proteolytic enzymes may cause consumption of α_2 -macroglobulin, thus explaining the low serum level. Such consumption has been reported in sepsis and acute pancreatitis when large amounts

of proteases are released (5,6).

The aim of the present study was to measure protease inhibitors in plasma and to characterize the dominating plasma protease inhibitors in faecal extracts with regard to functional activity and presence of protease-inhibitor complexes in two groups of patients, one with severe total colitis and the other with distal colitis.

Subjects, material and methods

Subjects: A series of twenty-six consecutive patients, admitted to the Department of Surgery, Malmö General Hospital, Malmö, Sweden, during a three-year period 1981-1983, was studied. On admission all patients had severe colitis according to the criteria of Truelove (7), and proctoscopy in combination with histological examination of rectal mucosa showed severe acute inflammation of the rectal mucosa. All patients were treated for a six-day period with parenteral hyperalimentation and intravenous administration of corticosteroids. In no case did emergency colectomy have to be performed.

All patients were closely followed and later divided into two groups according to the findings of colonoscopy with biopsies or in some cases radiological investigation by the double-contrast enema technique of the large bowel. These diagnostic procedures were completed within 4 months after the admission. Thirteen patients (6 men and 7 women) with a median age of 34 years (range, 22-67 years) had total colitis while in 13 patients (8 men and 5 women) with a median age of 24 years (range, 20-57 years) the disease was limited to the rectum and distal sigmoid (distal colitis). Radiological investigation with the double-contrast method showed the small bowel to be normal in all patients.

Blood samples were drawn on admission and placed into test-tubes containing EDTA. After centrifugation the plasma was separated and either immediately analysed or frozen at -20°C until analysed. Stool samples were collected within 12 hours

after admission and analysed fresh. Two grams of each stool sample were homogenized in 10 ml 0.9% NaCl at 4°C and extracted for two hours. The mixture was centrifuged at 39,000 x g for 30 minutes. The supernatant fluids were used for the analyses.

Controls: Eight healthy individuals (aged 25-50 years) without earlier or present signs of gastro-intestinal disorder were used as controls. The faecal samples were treated as described above.

Chemical material: Agarose, batch no. 61638, was purchased from Litex, Denmark. Bovine trypsin and chymotrypsin were obtained from Apoteksbolaget, Sweden, fibrinogen from AB Kabi, Sweden, Sephadex G-200 from AB Pharmacia Fine Chemicals, Uppsala, Sweden, N-alpha-benzoyl-DL-arginine-p-nitroanilide-hydrochloride (BAPNA) from BDH Chemicals Ltd, England, the chromogenic peptide substrates for α_2 -antiplasmin (S-2251), antithrombin III (S-2238) and granulocyte elastase (S-2484) from Kabi Diagnostica, Sweden and seronorm protein (batch no 102) from Nyegaard & Co, Oslo.

Antisera: Specific rabbit antisera to human α_2 -macroglobulin (α_2 -M), α_1 -protease inhibitor (α_1 -PI, formerly called α_1 -antitrypsin), antichymotrypsin (ACHY), cationic trypsin, chymotrypsin, pancreatic elastase 2, leucocyte neutral protease and leucocyte elastase were produced at the laboratory (8,9). Rabbit antisera to antithrombin III (AT III), α_2 -antiplasmin (α_2 -AP), inter-alpha-trypsin inhibitor (ITI), IgM and IgA were obtained from Professor C.B. Laurell, Department of Clinical Chemistry, Malmö.

Immunochemical methods: α_2 -M, α_1 -PI, ACHY, ITI, AT III, α_2 -AP and IgM were quantified by electroimmunoassay (10) using seronorm as a standard. The electrophoretic homogeneity of α_1 -PI and ACHY was studied by crossed immunoelectrophoresis (11). Samples with precipitation patterns indicating complex formation were further analysed by crossed immunoelectrophoresis with intermediary gel containing antisera to

human pancreatic proteases (cationic trypsin, chymotrypsin and elastase 2), leucocyte proteases (neutral protease and elastase) and IgA.

Enzymatic activity and inhibition assay: α_2 -AP and AT III in plasma were analysed with the chromogenic peptide substrates S-2251 (12) and S-2238 (13). The trypsin-inhibiting capacity of the faecal extracts was tested with BAPNA as a substrate (14) after adding increasing amounts of trypsin (0-10 μ g) to 100 μ l faecal extracts. The trypsin-binding capacity of α_1 -PI was also tested with crossed immunoelectrophoresis of faecal extracts before and after the addition of trypsin (0-10 μ g/100 μ l faecal extracts) with antiserum to α_1 -PI. The chymotrypsin-binding capacity of ACHY was tested with crossed immunoelectrophoresis of faecal extracts before and after the addition of chymotrypsin (0-10 μ g/100 μ l faecal extracts) with antisera to ACHY. Leucocyte elastase activity was analysed with S-2484 as a substrate according to the manufacturer's instructions. Non-specific proteolytic activity was tested on preheated fibrin agarose plates (15).

Gel-filtration: Faecal extracts (3 ml) were gel-filtrated on a Sephadex G-200 column (1.6x120 cm) at 4°C, equilibrated and eluted with 0.05M Tris HCl buffer, 0.5M NaCl, pH 7.4. The α_2 -M-containing fractions were pooled and concentrated 10 times by ultrafiltration (Amicon 52 with diaflo ultrafilter YM 10). The S-2484 splitting activity was measured before and after precipitation of α_2 -M with the γ -fraction of antisera to α_2 -M (16).

Statistics: Student's t-test (two-tailed) for unpaired observations was used. Values of $p < 0.05$ were considered significant.

Results

Plasma: The levels for α_1 -PI, α_2 -M, ACHY, ITI, α_2 -AP, AT III and IgM in patients with total and distal colitis are given in Fig. 1. All the patients had elevated plasma levels of the acute-phase reactants α_1 -PI and ACHY and no significant difference was noted between the two groups of patients. The α_2 -M level in plasma was significantly lower in patients with total colitis than in normal individuals and patients with distal colitis ($p < 0.001$). The levels of AT III and α_2 -AP were slightly elevated in both groups of patients and these two major inhibitors of plasmin and thrombin were measured both by immunochemical and enzymological methods with identical results. The plasma levels of ITI and IgM were normal in all patients. No protease α_1 -PI complex formation could be demonstrated in plasma by immunoelectrophoresis.

Faecal extracts: The same recovery was obtained for immuno-reactive α_1 -PI, α_2 -M and ACHY after dilution of the plasma samples and faecal extracts (parallel dilution curves) indicating similar immunoreactivity for these proteins in plasma and faecal extracts. After gel-filtration of faecal extracts, α_2 -M, α_1 -PI and ACHY eluted in volumes corresponding to the native proteins. In some samples, however, a small additional α_1 -PI peak was demonstrated eluting in a volume corresponding to protease- α_1 -PI complexes (Fig. 2). In faecal extracts from normal individuals α_2 -M and ACHY could not be detected, and only very small amounts of α_1 -PI were found. The levels of α_1 -PI, α_2 -M and ACHY in faecal samples from patients with acute total or distal colitis are shown in Table I.

In the pooled and concentrated α_2 -M-containing fractions obtained with gel-filtration, an enzymatic activity against S-2484 corresponding to 3.5 μ g (1.7-4.7 μ g) leucocyte elastase/mg α_2 -M was found. Ninety per cent of the activity was precipitated using antibodies to α_2 -M.

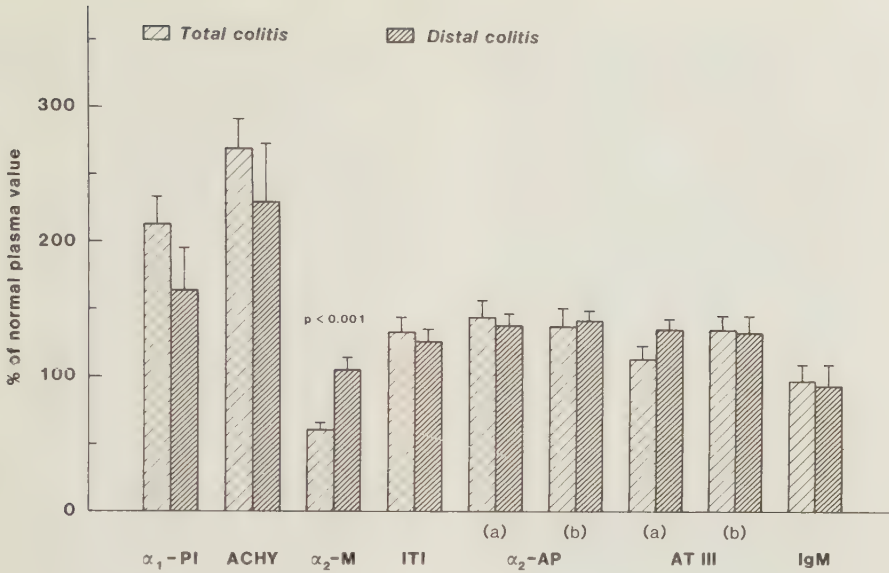


Fig. 1 Plasma levels expressed as per cent of the standard used measured with electroimmunoassay of α_1 -protease inhibitor (α_1 -PI), antichymotrypsin (ACHY), α_2 -macroglobulin (α_2 -M), inter-alpha-trypsin inhibitor (ITI), α_2 -antiplasmin (α_2 -AP), antithrombin III (AT III) and IgM in 13 patients with total colitis and 13 patients with distal colitis. α_2 -AP and AT III are measured both with electroimmunoassay (a) and low-molecular-weight substrate (b). Mean values $\pm$ 1 SD.

Table I

Protease inhibitors in faecal extracts from patients with total colitis, distal colitis and normal subjects, expressed as mg/g faeces wet weight. Median and range are given (n.d. = not detectable).

	N	α_1 -PI	α_2 -M	ACHY
Total colitis	13	0.42 (0.06-0.58)	0.09 (0.04-0.4)	0.14 (0.01-0.29)
Distal colitis	13	0.15 (0.02-0.33)	0.02 (0.01-0.24)	0.02 (0.01-0.09)
Normal	8	0.02 (n.d.-0.05)	n.d.	n.d.

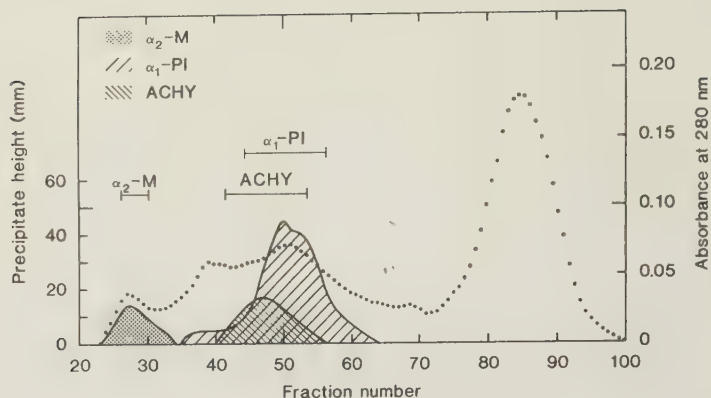


Fig. 2 Elution profile of α_2 -macroglobulin (α_2 -M), α_1 -protease inhibitor (α_1 -PI) and antichymotrypsin (ACHY) on a Sephadex G-200 column (1.6x120 cm) eluted with 0.05 M Tris-HCl buffer 0.5 M NaCl, pH 7.4. Absorbance measured at 280 nm. α_2 -M, α_1 -PI and ACHY were measured with electroimmunoassay. The elution volumes for native α_2 -M, α_1 -PI and ACHY are indicated at the top of the figure. α_2 -M and ACHY eluted in volumes corresponding to the native proteins. α_1 -PI eluted also in a volume corresponding to protease- α_1 -PI-complexes.

Characterization of immunoreactive α_1 -PI in faecal extracts with crossed immunoelectrophoresis showed only one peak in the majority of samples. Electrophoretically this peak migrated slightly more slowly than the free native α_1 -PI (Fig. 3). In some samples two additional peaks, (a) and (b), were seen in the β - and γ -regions, respectively (Fig. 3B). These later samples also showed a small additional α_1 -PI peak on gel-filtration indicating complexation. They were further analysed with crossed immunoelectrophoresis with intermediary gel containing antisera against the different leucocyte proteases, pancreatic proteases and IgA. Complex formation could be demonstrated between α_1 -PI and the leucocyte proteases, elastase and neutral protease (Fig. 4) and also with IgA. No complex formation between α_1 -PI and pancreatic proteases was detected. Characterization of ACHY in faecal extracts with

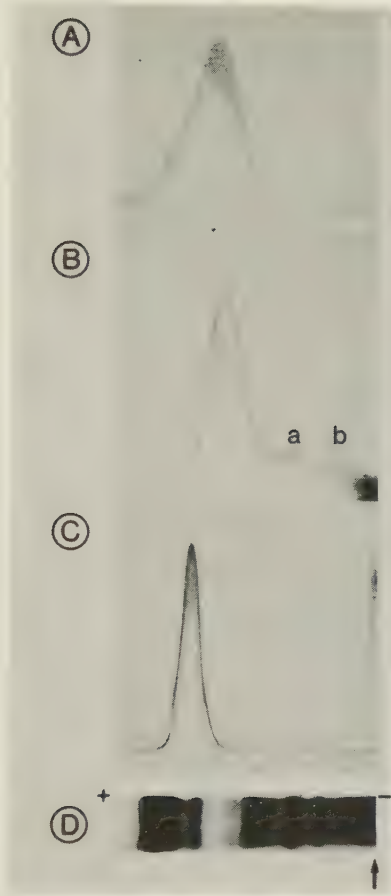


Fig. 3 Precipitation patterns obtained from crossed immunoelectrophoresis of faecal extracts (A,B) and plasma (C) from patients with colitis, using rabbit antiserum to human α_1 -PI. Agarose gel electrophoresis of human plasma is given as a reference (D). The arrow indicates the application point.

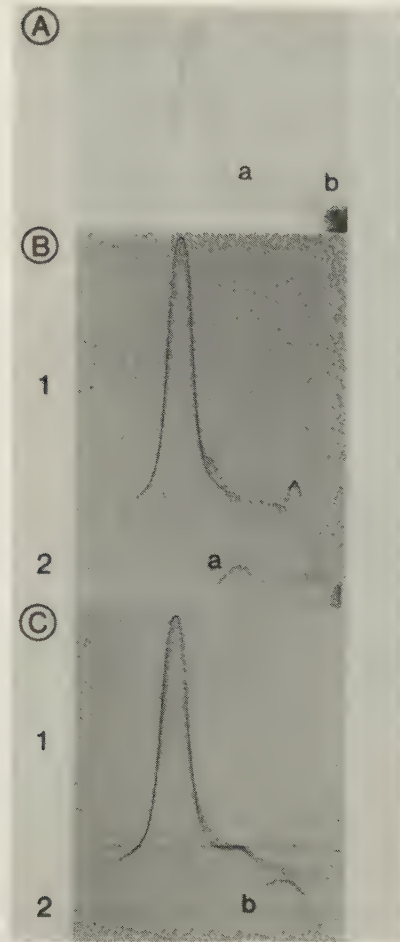


Fig. 4 Precipitation patterns obtained from crossed immunoelectrophoresis with antisera to α_1 -PI (A,B1,C1) of faecal extracts using intermediary gels containing antisera against neutral protease (B2) and leucocytic elastase (C2). The peak (a) of α_1 -PI in complex was due to neutral protease, since it was specifically eliminated by antibodies to this enzyme. With the same reasoning, peak (b) was due to leucocytic elastase- α_1 -PI complex.

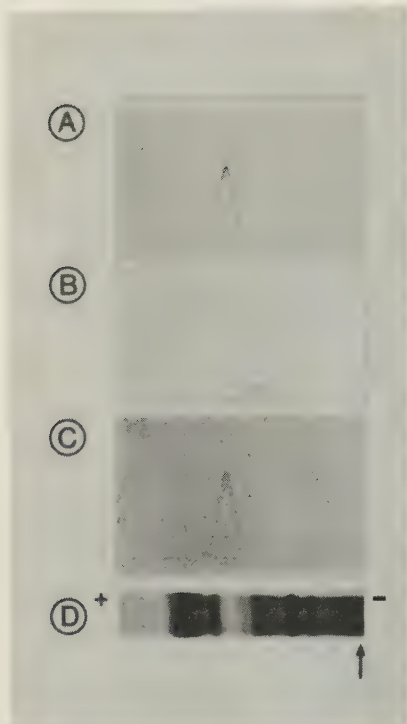


Fig. 5 Precipitation patterns obtained from crossed immunoelectrophoresis of faecal extracts A and B and plasma C from patients with colitis, using rabbit antisera against human antichymotrypsin (ACHY). Agarose gel electrophoresis of human plasma is given as a reference (D). The application point is indicated with an arrow.

crossed immunoelectrophoresis showed only one peak. Electrophoretically this peak migrated more slowly in some samples than the free native ACHY (Fig. 5).

No residual trypsin-inhibiting capacity could be demonstrated in faecal extracts. In fact, all but two of the extracts showed BAPNA cleaving activity as well as non-specific proteolytic activity even before the addition of trypsin. Furthermore, the addition of trypsin or chymotrypsin to faecal extracts did not cause any change in the α_1 -PI or ACHY crossed immunoelectrophoretic precipitation patterns.

Discussion

Although the plasma level of α_2 -M is remarkably stable under most conditions, low levels have been reported in sepsis (5) and in acute pancreatitis (6). These diseases are characterized by a release of large amounts of proteases from leucocytes and the pancreatic gland. The low plasma α_2 -M levels

are thought to be the result of a consumption and elimination of α_2 -M after complex formation with proteases (17). A similar mechanism might exist in acute colitis as judged from our demonstration of complexes between leucocyte proteases and α_2 -M as well as α_1 -PI in faecal extracts. The slightly increased plasma levels of functionally active α_2 -AP and AT III in plasma argue against any substantial consumption of α_2 -M by plasmin and/or thrombin release in colitis.

It is difficult to measure and characterize proteases bound by α_2 -M with immunochemical techniques. Small peptides like S-2484 acting as specific substrates are, however, cleaved by enzymes captured within the huge inhibitor molecule (18). Following gel-filtration of faecal extracts, we could demonstrate leucocyte elastase-like enzymatic activity in the α_2 -M-containing fractions. The activity was almost totally precipitable by antibodies against α_2 -M, indicating complex formation between α_2 -M and leucocyte elastase. Earlier in vitro studies have demonstrated that when leucocyte elastase is added to serum samples 90 per cent is complexed to α_1 -PI and 10 per cent to α_2 -M (19). Corresponding figures for the leucocyte neutral protease are 50 per cent to α_1 -PI and 50 per cent to α_2 -M (20). Thus, the finding of α_1 -PI in complex with leucocyte proteases in the faecal extracts is also evidence of the binding of these proteases, elastase as well as neutral protease, to α_2 -M in such extracts. The complexes demonstrated in the faecal extracts probably only give a weak reflection of the protease-protease-inhibitor interaction taking place in the intestinal wall. Protease- α_2 -M complexes formed in the tissue fluids are largely eliminated by macrophage-like cells in the local tissues and regional lymph nodes (21). The magnitude of the processes is probably best indicated by the low α_2 -M levels in plasma.

The plasma level of IgM which is a molecule of similar size as α_2 -M (molecular weight about 900,000 and 725,000 daltons, respectively) was within normal limits as has earlier been reported in patients with quiescent or acute attacks of inflammatory bowel disease (2,3). Haemodilution or increased

loss of macromolecules through the intestinal wall thus seems a less likely major cause of the low α_2 -M plasma levels found in the patients with total colitis. No attempts were made to estimate the intestinal losses of the different protease inhibitors from the results of direct measurements in faeces as pretreatment faecal samples were obtainable only during the first few hours after the admittance to the hospital. The faecal levels of the protease inhibitors are therefore presented in mg/g wet weight of faeces only to give an idea of the concentration range.

The changed electrophoretic mobility of non-complexed α_1 -PI in faecal extracts probably represents a destruction of the inhibitor or, as has earlier been discussed, represents the inhibitor partner of previous complexes with leucocyte proteases (22). The abnormal behaviour of these inhibitor molecules was further demonstrated by the finding that the addition of active trypsin to the faecal extracts did not result in further complex formation of α_1 -PI. Similarly, faecal ACHY was non-reactive. The absence of any residual protease-inhibiting capacity was, in most faecal extracts, accompanied by the presence of active proteases. As the extracts contain material from the exudates of the inflamed intestinal mucosa, these data probably indicate a local protease-antiprotease imbalance in active ulcerative colitis.

The data obtained in the present study confirm the observation that severe total colitis, but not distal colitis, is characterized by low plasma levels of α_2 -M. Furthermore, the data presented indicate that the release of leucocyte proteases may account for a consumption of α_2 -M as has been demonstrated earlier in acute pancreatitis and sepsis. The absence of residual protease inhibiting capacity in the intestinal discharges indicates a local protease-antiprotease imbalance in the inflamed intestinal mucosa.

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References

1. Bicks RO, Kirsner JB, Palmer WL. Gastroenterology 1959,37,256-262
2. Weeke B, Jarnum S. Gut 1971,12,297-302
3. Bendixen G, Goltermann N, Jarnum S, Jensen KB, Weeke B, Westergaard H. Scand J Gastroent 1970,5,433-441
4. Brown DJC, Khan JA, Copeland G, Jewell DP. J Clin Lab Immunol 1980,4,53-457
5. Gallimore MJ, Aasen AO, Smith Erichsen N, Larsbraaten Lyngaas K, Amundsen E. Thromb Res 1980,8,601-608
6. McMahon MJ, Bowen M, Mayer AD, Cooper EH. Am J Surg 1984,147,164-170
7. Truelove SC, Witts LJ. Br Med J 1955,2,1041-1048
8. Balldin G, Ohlsson K. Surgery 1979,85,451-466
9. Bohe M, Borgström A, Genell S, Ohlsson K. Digestion 1983,27,8-15
10. Laurell CB. Scand J Clin Invest 1972,124,21-37
11. Ganrot PO. Scand J Clin Lab Invest 1972,124,39-47
12. Teger-Nilsson A-C, Friberger P, Gyzander E. Scand J Clin Lab Invest 1977,37,403-409
13. Ödegård OR, Lie M, Abildgaard U. Thromb Res 1975,6, 287-294
14. Erlanger BF, Kokowsky N, Cohen W. Arch Biochem 1961,95,271-278
15. Lassen M. Acta Phys Scand 1952,27,371-376
16. Genell S, Ohlsson K, Wehlin L. Hoppe-Seyler's Z Physiol Chem 1979,358,115-121
17. Ohlsson K, Laurell C-B. Clin Sci Mol Med 1976,51,87-92

18. Starkey PM, Barrett AJ. In: Barrett AJ, ed. *Proteinases in Mammalian Cells and Tissues*. Elsevier/North Holland Biomedical Press, 1977,663-693
19. Ohlsson K, Olsson I. *Scand J Clin Lab Invest* 1974,34, 349-355
20. Ohlsson K, Olsson I. *J Lab Clin Med* 1977,89,269-277
21. Ekerot L. *Scand J Plast Reconstr Surg* 1982,16,107-115
22. Tegner H. *Acta Otolaryngol*,1978,85,282-289

